THE RESTING AND EXCITED STATES OF BIOLOGICAL MEMBRANES

By

James Bradley, B.Sc.

A Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh. October, 1966.

C O N T E N T S

Page

SYMBOLS AND ABBREVIATIONS

A_n, A_m : constants proportional to the permeability of the nth and mth ions.

B_n : a constant equal to the nth ion transport number.

C : capacitance per unit thickness of membrane (μF. cm^{-1}.).

C_n^1, C_n^2 : concentrations of nth ion in phases 1 and 2 (mM l^{-1}.).

C_n^i, C_n^o : internal and external concentrations of the nth ion (mM l^{-1}.).

C_m : membrane capacitance (μF. cm^{-2}., unless specified).

d : membrane thickness (cm.).

E : membrane potential (mV).

E_c : command potential (mV).

E_e : excited state potential (mV).

E_m : resting potential (mV).

E : resting potential displacement (mV).

g_n : conductance of the nth ion.

I : membrane current (μA. cm^{-2}., unless specified).

I_n : current carried by the nth ion (μA).

$_1J_2^n$, $_2J_1^n$: fluxes of nth ion between phases 1 and 2 (moles/cm^2.sec.).

k_n : nth ion partition coefficient.

P_n : permeability of nth ion (cm.sec^{-1}.).

R : clamp control resistance (KΩ).

R : mean membrane resistance (KΩcm^2.).

R_e : membrane excited state resistance (KΩcm^2.).

R_i : clamp internal resistance (KΩ).

R_m : resting membrane resistance (KΩcm^2.).

R_m' : membrane resistance (KΩ).

R_s : electrode resistance (KΩ).

R_x : short-circuit control resistance (KΩ).

$R(x)$: resistivity of membrane (KΩcm.)

r_i : resistance of cell interior (KΩcm.$^{-1}$).

r_m : resistance of unit length of cell membrane (KΩcm.).

r_o : resistance of external solution (KΩcm.$^{-1}$).

T_n : transport number of nth ion.

u_n : mobility of nth ion (cm.^{2}/volt sec.).

V : potential between Ag/AgCl electrodes (mV).

V_n : chemical potential difference expressed in mV.

V_o : membrane potential displacement at x = 0 (mV).

V_s : short-circuit E.M.F.

V_x : membrane potential displacement.

Z_n : valency of nth ion.

Z_m : membrane impedance.

ϕ_n : flux of nth ion (moles/cm.^{2}sec.).

ψ^1 : potential of phase 1 (mV).

λ : space constant (cm.).

τ_c : membrane time constant (msec., unless specified).

τ_d : redistribution time constant (sec.).

$\bar{\mu}^1$: electrochemical potential of an ion in phase 1 (joules/mole).

μ_o^1 : standard chemical potential of an ion in phase 1 (joules/mole).

μ : feedback amplifier amplification factor.

C H A P T E R I

GENERAL INTRODUCTION

1.1 INTRODUCTION

In recent years, a vast body of information has been accummulated on the electrical properties of biological membranes. It has been found that electrical potential differences occur across them, that ions can penetrate them and that they have a quite high resistance to the flow of electric current through them. Analysis of this information has revealed that there is no general theory of membrane phenomena which can explain all of it. As well as these general phenomena, some cell membranes, particularily those of nerve cells, are capable of producing a temporary and localised change in membrane potential and membrane resistance, which is propagated along the membrane as an electrical impulse.

An excitable membrane is nowadays regarded as being one of the basic functional units in a biological computer or control system and in view of its fundamental importance, it is hardly surprising that probably more information is available on the electrical properties of nerve membranes (particularily the giant nerve from the squid) than on any other membrane. A great deal is now known about the action potential, as the phenomenon of exitability is called, and despite the illuminating hypothesis of Hodgkin and Huxley, the problem of the exact physical chemical mechanism of excitability remains to be solved.

All nerves are capable of conducting impulses and so are muscle cells; so also are certain giant plant cells of the Characeae. Over the years the development of knowledge about

the action potential in nerve has proceeded side by side with the study of the action potential in these plant cells and in this thesis the giant plant cell Nitella translucens has been chosen for studying biological membranes in both their resting and excited states. This chapter is concerned mainly with the theoretical basis of electrical phenomena in membranes in general, and with the electrical properties of biological membranes in particular.

1.2 THE ELECTRICAL PROPERTIES OF MEMBRANES

1.21 The Nature of the Membrane Potential

When two aqueous solutions of electrolytes are separated by a membrane, in general an electrical potential difference exists between the two solutions. This potential difference is a diffusion potential in the widest sense and is caused by transport of ions or electrons through the membrane at different rates.

The simplest kind of membrane potential is the Donnan potential. This occurs when the membrane separating the two solutions allows only a single ionic species to pass through it. This permeable ion will have a tendency to diffuse across the membrane from the region of high concentration to the region of low concentration. This will cause each solution to acquire equal and opposite charges so that the resulting electric field through the membrane will be sufficient to prevent any net transport of charge. Thus no diffusion can occur in the steady state. However, ions of the permeable species will continue to move across the membrane, but ion fluxes in opposite directions will be equal. When a Donnan equilibrium exists, an ion which traverses the membrane will acquire or release no energy; thus the sum of the electrical potential and chemical potential energy difference between the two solutions must be zero, so that:

$$\bar{\mu}' - \bar{\mu}^2 = 0 \qquad (1.1)$$

or: $$\mu_0' - \mu_0^2 + RT \ln \frac{c'}{c^2} + zF(\psi' - \psi^2) = 0 \qquad (1.2)$$

so that approximately: $$\Delta_2 \psi = \frac{RT}{zF} \ln \frac{c'}{c^2} \qquad (1.3)$$

where $\bar{\mu}$ is defined as the electrochemical potential of an ion; μ_o is the standard chemical potential of that ion, ψ is its electrical potential and C is its concentration. The superscripts 1 and 2 refer to the solutions. Equation (1.3) is known as the Nernst equation and if it holds for an ion, then that ion is said to be in electrochemical equilibrium between the two phases and there can be no net flux of that ion through the membrane, always assuming, of course, that $(\mu_o' - \mu_o^2)$ may be neglected.

In general, the membrane potential is due to the membrane being permeable to more than one ionic species. In this case, net transfer of ions and salt may occur. The two phases separated by the membrane become charged up, so that the resulting electric field across the membrane is capable of preventing net transport of charge, while permitting net transport of ions. The exact expression for the relation between the membrane potential, the concentrations of ions in the phases and the properties of the membrane is very involved and not very useful. It was first derived by Nernst (1888) and Planck(1890). However, the situation may be handled to a good approximation by simple theory.

It has been mentioned that the diffusion potential is due to ions diffusing at different rates, which amounts to stating that it is due to the membrane having differential permeabilities to ions. For a given ionic species, there must be two extreme cases: either it is the only permeable ion or it is not permeable at all. Hence, if it is the only permeable ion, then the membrane potential is given by equation (1.3) and if it

is not permeable, then it can have no effect on the membrane potential. Thus it is clear that any general equation relating membrane potential and ion concentrations must be such that if any single ionic species is considered, the equation will reduce to one in which the membrane potential is independent of the concentrations of the selected species of ion when that ion is impermeable, and further, it will reduce to the Nernst equation whenever all ionic species other than the selected species become impermeable. The form of the Nernst equation is such that two basic empirical expressions for the membrane potential are possible. These are:

$$ {}_1\Delta_2\psi = \frac{RT}{F} \ln \frac{\sum_n A_n C_n^{+2} + \sum_m A_m C_m^{-1}}{\sum_n A_n C_n^{+1} + \sum_m A_m C_m^{-2}} \qquad (1.4a) $$

$$ {}_1\Delta_2\psi = \sum_n \frac{RT}{Z_n F} B_n \ln \frac{C_{n_2}}{C_{n_1}} \qquad (1.5a) $$

where the subscripts n and m refer to the ionic species and A_n, A_m and B_n are constants. In equation (1.4a) distinction has to be made between positive and negative ion concentrations. The constants A_n may have any units, while the constants B_n must have no units and must always sum to unity.

In the study of bioelectric phenomena, equations (1.4a) and (1.5a) are extensively used in the form of equations (1.4b) and (1.5b):

$$ {}_1\Delta_2\psi = \frac{RT}{F} \ln \frac{\sum_n P_n C_n^{+2} + \sum_m P_m C_m^{-1}}{\sum_n P_n C_n^{+1} + \sum_m P_m C_m^{-2}} \qquad (1.4b) $$

$$ {}_1\Delta_2\psi = \sum_n \frac{RT}{Z_n F} T_n \ln \frac{C_{n_2}}{C_{n_1}} \qquad (1.5b) $$

where P_n is defined as the membrane permeability, measured in

the somewhat arbitrary units of cm.sec^{-1}. The T_n are referred to as the ionic transport numbers of the membrane, but these are not in general equal to the true electrical transport numbers (see Finkelstein and Mauro, 1963). Both of these equations can be derived from the Nernst-Planck equations, which state that the flux of an ion across a membrane is equal to the ionic mobility times concentration times the electrochemical potential gradient:

$$\phi_n = u_n C_n \frac{d\bar{\mu}_n}{dx} \qquad (1.6)$$

Goldman (1943) integrated these equations by assuming a constant electric field across the membrane and equation (1.4b) is known as the Goldman equation. Hodgkin ~~and Huxley~~ (1957) first derived equation (1.5b) using an electrical analogue, but it was later deduced from the Nernst-Planck equations by Finkelstein and Mauro (1963). It is called the transport number equation and involves no special assumptions; the T_n are, however, left in integral form.

The membrane potential is normally measured by means of salt bridge electrodes immersed in the solutions separated by the membrane and connected to a high input impedance voltmeter. The potential thus measured is the sum of the membrane potential, the diffusion potentials of the salt bridges and any diffusion potentials across the unstirred layers of solution which can exist on either side of the membrane. If the salt bridges are properly designed, diffusion potentials will be zero or at worst will be opposite and cancel, while potentials across any unstirred layers will be negligible in the steady state provided the resistivity of the membrane is greater than that of the bulk solutions.

1.22 The Membrane Resistance

If an electric current is passed across a membrane separating two aqueous solutions, then in general the potential difference between the two solutions will change. Using the Nernst-Planck equations and writing the fluxes as currents, Planck (1890) originally derived the relation:

$$\Delta_2\psi = I\int_1^2 \frac{dx}{F^2\left(\sum_n u_n C_n^+ + \sum_m u_m C_m^-\right)} + \frac{RT}{F}\int_1^2 \frac{d\left(\sum_n u_n C_n^+ - \sum_m u_m C_m^-\right)}{\left(\sum_n u_n C_n^+ + \sum_m u_m C_m^-\right)} \quad (1.7)$$

where I is the current passing across the membrane. When I is zero the membrane potential is given by:

$$\Delta_2\psi = \frac{RT}{F}\int_1^2 \frac{d\left(\sum_n u_n C_n^+ - \sum_m u_m C_m^-\right)}{\left(\sum_n u_n C_n^+ + \sum_m u_m C_m^-\right)} \quad (1.8)$$

and this is just an integral form of equations (1.4b) and (1.5b). Thus the expression:

$$R_m = \frac{1}{F^2}\int_1^2 \frac{dx}{\left(\sum_n u_n C_n^+ + \sum_m u_m C_m^-\right)} \quad (1.9)$$

gives the membrane resistance in integral form. Equation (1.7) is just a statement of the fact that when a current is passing, the membrane potential is made up of an IR potential difference plus what is often referred to as the resting potential. Thus it is clear that the membrane may be represented by a battery of E.M.F. equal to the resting potential, and internal resistance equal to the membrane resistance.

The membrane resistance will be independent of the applied current and constant in time only if the ionic concentration profiles in the membrane are not affected by the applied current; in general, the ions in the membrane always redistribute themselves when current is applied (see Cole, 1965) so that the membrane resistance will be both frequency and amplitude

dependent. However if the redistribution time constant is long compared with the membrane time constant, then the membrane resistance as given by equation (1.9) will be sensibly constant. Transient membrane phenomena will be discussed in more detail later.

1.23 Fluxes, Conductances and Permeabilities

The flux of an ion across a membrane is given by the Nernst-Planck equations. Partial integration of these equations gives:

$$I_n = \left\{ \int_1^2 \frac{dx}{F^2 u_n C_n} \right\}^{-1} \left\{ \Delta_2 \psi + \frac{RT}{Z_n F} \ln \frac{C_{n_1}}{C_{n_2}} \right\} \qquad (1.10)$$

and this equation may be rewritten (Finkelstein and Mauro, 1963) in electrical terminology as: $I_n = g_n (E + V_n)$ (1.11)

where I_n is the flux of the ion across the membrane measured in units of electric current, g_n is the membrane conductance of the ion, E is the electrical potential difference across the membrane and V_n is the chemical potential difference across the membrane measured in units of electrical potential. It is to be noted that I_n, E and V_n are each physically determinable, so that g_m is defined by equation (1.11). Since (1.11) is true for each diffusing ionic species it is possible to construct an equivalent circuit to represent the behaviour of a membrane permeable to ions (see Fig. 1.1). Each arm of the analogue is associated with a particular ionic species; the flux of an ionic species is thus determined by the current in the appropriate arm, allowance being made for the sign of the charge on the ion. If the I_n are eliminated from equations (1.11) then:

$$E = -\sum_n \frac{g_n}{(\sum_n g_n)} V_n \qquad (1.12)$$

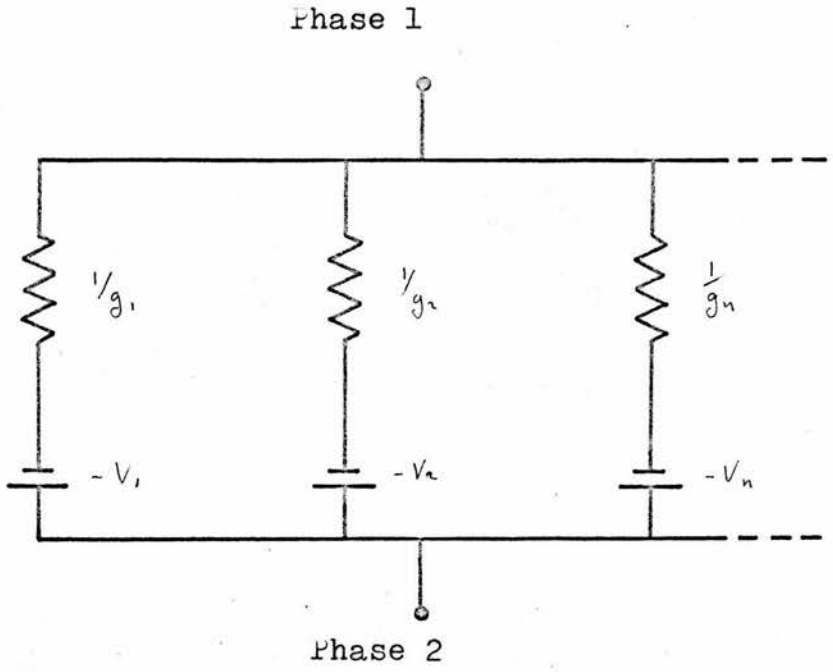


Fig. 1.1 A simple electrical analogue of a membrane. The symbols are explained in the text.

and this was the original method of deriving the transport number equation. The transport number of an ion is thus equal to $\frac{g_n}{\sum_n g_n}$ according to the equivalent circuit. However, this transport number is not equal to the true electrical transport number of an ion, which is the fraction of applied membrane current carried by that ion. This is evident from the inequality:

$$\left\{ \int_1^2 \frac{dx}{F^2(\sum_n u_n C_n^2 + \sum_m u_m C_m^2)} \right\}^{-1} \neq \sum_{n,m} \left\{ \int_1^2 \frac{dx}{F^2 u_n C_n} \right\}^{-1} \qquad (1.13)$$

which is a statement of the fact that $\sum_n g_n$ is not equal to the membrane conductance. This fact is not generally realised but was pointed out recently by Finkelstein and Mauro (1963). Matters are greatly simplified if $\sum_n g_n$ is equal to the total membrane conductance and if this is assumed no great error is likely to be involved provided the distributions of ionic concentrations through the membrane are not assymetric. Despite this difficulty, the concept of an ionic membrane conductance is a very useful one and is widely used in the study of bioelectric phenomena.

By assuming a constant electric field across the membrane, Hodgkin and Katz (1949) have integrated the Nernst-Planck equations to give the relation between the flux of an ion and its permeability:

$$\phi_n = - P_n \frac{z_n F E / RT}{1 - \exp z_n F E / RT} \left\{ C_n^2 - C_n^1 \exp z_n F E / RT \right\} \qquad (1.14)$$

where the permeability P_n is defined according to:

$$P_n = \frac{u_n R T k_n}{d} \qquad (1.15)$$

k_n is the ion partition co-efficient between the membrane and the solution and d is the membrane thickness. Equation (1.14)

is not so widely used as (1.11) because of its complicated form and dubious assumption of a constant electric field across the membrane.

When an ionic species is in equilibrium or is diffusing across a membrane there is a flux of ions in both directions across that membrane. Any net ion flux is the difference between these two fluxes so that: $\phi_n = {}_1J_2^n - {}_2J_1^n$ (1.16)
Ussing (1949) and Teorell (1949) have shown that:

$$ {}_1J_2^n / {}_2J_1^n = \exp\left(\bar{\mu}_n^1 - \bar{\mu}_n^2\right) \qquad (1.17) $$

This equation was deduced on quite general grounds and when ${}_1J_2^n$ is equal to ${}_2J_1^n$ it reduces to the Nernst equation. Measurement of these fluxes is normally accomplished by the use of radioactive tracers.

1.24 Electrical Transients in Membranes

Transient electrical phenomena in membranes are due mainly to the membrane capacitance and to ionic redistribution effects. Following the application of a constant current to the membrane, excess charge builds up on either side of the membrane and this produces the changing electric field. As the membrane potential grows, more charge passes through the membrane and less is used to charge up the two phases separated by the membrane. The growth of the membrane potential will be exponential and is given by: $E = I R_m \left(1 - \exp \frac{t}{\tau_c}\right)$ (1.18)
where τ_c is the membrane time constant, which is equal to $R_m C_m$. This is a method of defining the membrane capacitance C_m. Equation (1.18) was originally derived by Planck (1890) for a uniform electrolyte solution. However, it is not quite correct

to apply this equation to a membrane, since in the general case the membrane conductivity varies throughout the membrane. Thus, a simple relaxation with a single time constant is not in theory sufficient to describe a capacitance transient in a membrane, (see Cole, 1965). The membrane impedance is more accurately given by:

$$Z_m = \int_1^2 \frac{R(x)\,dx}{(1 + i\omega C R(x))} \qquad (1.19)$$

where R(x) is the membrane resistivity at any point in the membrane and C is the capacitance per unit thickness of membrane. Thus the time constant is more correctly described by $\bar{R}C_m$, where $\bar{R}$ is the mean resistance of the membrane; however, this is still an approximation. In spite of these difficulties, a membrane may be represented by a battery and resistance in series with a capacitance; this has been found to be adequate for most experimental purposes.

When the capacitance charging process is completed, in general a current is flowing across the membrane. Since, as already stated, $\sum_n g_n$ is not equal to the total membrane conductance, at any given point in the membrane the flux of an ion will be different from that at any other point in the membrane. While this is happening the membrane is in a steady state. Due to the unequal flow of ions at different points in the membrane however, the principle of continuity requires that the concentrations of ions in the membrane begin to change. It may take a long time for these concentration changes to appear, but when they do the membrane is no longer in a steady state but in a transient state. The changing of the state of the

membrane during this period is known as the redistribution process. Changes in ion concentrations will proceed everywhere until a time is reached when the flow of each ion is the same at all points in the membrane. When this happens the membrane is in a true steady state. Incidently in this state $\sum_n g_n$ is equal to the total membrane conductance so that the equivalent circuit representation of the membrane is quite correct; however, each of the membrane g_n in this steady state is different from the corresponding g_n of the membrane before the prolonged application of current. Because of the changes in ionic concentrations in the membrane during this process, both the membrane resistance and the individual g_n change so that the relation between the membrane potential and applied current is non-linear. If the membrane resistance increases during the redistribution process then the magnitude of the membrane potential will also increase, and if the resistance decreases then the potential will decrease.

This transient redistribution potential has been investigated by Cole (1965) and he estimates that the time constant of the effect is approximately given by: $\tau_D = \frac{d^2}{\pi^2 u k T}$ (1.20)

The redistribution effect may also overlap with the charging effect and in this case the effects may be resolved only by measuring the membrane impedance over a relevant frequency range. In conclusion transient membrane phenomena are rather difficult, but their study can yield information about membrane properties.

1.25 Special Classes of Membranes

The most general type of membrane is the homogeneous membrane

and it may be regarded as a membrane which does not exhibit any macroscopic variation within any plane parallel to the surface. It has no special properties.

A mosaic membrane (Finkelstein, 1964) is usually regarded as one which consists of a parallel array of regions which are permselective to different ionic species. This membrane has the property that $\Sigma_n g_n$, as obtained from flux measurements, is equal to the total membrane conductance. Further the potential may be exactly predicted by the transport number equation, the T_n being constants.

Fixed charge membranes have been investigated by Teorell (1953) and Vaidhyanathan (1965). These membranes contain stationary charges in the membrane structure. If the concentration of an ion in one of the solutions is decreased, this ion will tend to be exchanged out of the membrane thus decreasing the membrane conductance of that ion. Hence stepwise decrease of the concentration of an ion will have a gradually decreasing effect on the membrane potential; similarily stepwise increase of the concentration of an ion will have a steadily increasing effect on the membrane potential.

A redox membrane is permeable to electrons as well as ions. Electron transport will occur when it separates two solutions which contain redox components. The behaviour of this membrane may be adequately predicted by the electrical analogue (Fig. 1.1) using for the electron battery the thermodynamic expression for the free energy released or stored per equivalent of electrons transported across the membrane. When current is passed across this membrane, some of it will be carried by electrons, and so

the membrane resistance cannot be deduced from ion fluxes alone.

An electrogenic or 'fuel-cell' membrane is capable of converting chemical energy into electrical energy directly. It is assymetric and consumes the oxidant (for example O_2) on one side and the fuel on the other. In general, it can also be permeable to ions. Thus the membrane may be most simply described in terms of the electrical analogue in Fig. 1.1, if one of the battery voltages depends only on the free energy released per equivalent of oxidant consumed. If such a membrane separates two equal solutions, there will be a potential difference across it and if this potential is short-circuited then the current will be equal to the Faraday constant times the number of equivalents of oxidant consumed per second. Membranes operating on these principles have been constructed in recent years (Phillips, 1962) and are used as power supplies.

A composite membrane consists of two or more individual membranes in series. At present the most interesting composites involve anion and cation exchange membranes (Mauro, 1962; Coster, 1965). Mauro and Coster have shown that the junction between a cation and anion exchange membrane (a c-a junction) exhibits the same kind of rectifying properties as the semiconductor p-n junction, and for the same reasons. Coster has also shown that a thin c-a junction would exhibit reversible break-down effects similar to the effects in p-n junctions. It now seems likely that many semiconductor phenomena can occur in systems made up of cation and anion exchange material (c-type and a-type material).

1.3 SOME PROPERTIES OF BIOLOGICAL MEMBRANES

1.31 Membrane Structure.

Studies of the penetration of the plasma membrane by a variety of solutes has shown that the permeability of a solute is broadly determined by its ability to penetrate a thin layer of oil. Fricke (1933) studied the behaviour of cell suspensions in an alternating current and concluded that the plasma membrane was a relatively non-conducting layer of the order of 50A thick. This early estimate of the thickness of a biological membrane has been confirmed by recent investigations with the electron microscope, (Robertson, 1957).

A great many attempts have been made to build up a picture of the molecular structure of the plasma membrane from the known properties of proteins and lipids. In 1925, Gorter and Grendel spread lipid extracted from red blood cells as a unimolecular film on the surface of a Langmuir trough, and from a knowledge of the total surface area of the cells from which the extract was taken, they concluded that the lipid in the plasma membrane was ordered in what has now come to be called a 'bimolecular leaflet'. Later, Grendel (1929) analysed the lipid extract and showed that the leaflet was about 30A thick. The lipid composition of membranes varies but cholesterol and phospholipids are the main constituents. Gorter and Grendel proposed that this bimolecular membrane was arranged with the hydrocarbon ends of the lipids on the inside and the polar groups on the outside. Danielli and Harvey (1935) and other workers measured the surface tension of eggs and other cells and found it to be of a much lower order than that of a simple lipoid surface and they concluded that

this indicated the presence of a surface active material such as protein. Since proteins are capable of spreading out in films, even on the surface of an existing lipoid film whose stability will, as a result, be markedly enhanced, Davson and Danielli (1952) have proposed that the general basic pattern of membrane structure is a double layer of lipid with a layer of protein at each interphase. The minimum thickness of this membrane would then be about 80A. Very recently, purified lipid bimolecular membranes have been successfully formed between two aqueous solutions (Huang, Wheeldon and Thompson, 1964) but measurement of their electrical resistance indicates that they are about 10^2 times less permeable to ions than the membranes of living cells.

1.32 Biological Resting Potentials

Where it has been possible to make the measurements, it has been shown that between the interior of an individual cell and its environment there exists an electrical potential difference. It is possible to insert a fine salt bridge electrode into the interior of large single nerve fibres, muscle fibres and certain giant plant cells and by connecting this electrode through a suitable electrometer to another salt bridge in the surrounding medium, potential differences of up to 150 mV may be recorded, (Ling and Gerard, 1949; Graham and Gerard, 1946 and Walker, 1955). The cell is not damaged if this insertion is carefully carried out and the potential recorded will remain constant for many hours. Electrical potential differences also occur across certain membranous tissues consisting of parallel layers of cells; the most widely studied is frog skin but toad and gall bladder have also received a great deal of attention.

The electric organs of certain fishes have also been studied in some detail; the electric eel, Electrophorus electricus, can, on open circuit, produce a discharge of about 600 volts with a power output of about 100 watts. This electric organ consists of a very large number of compartments or 'electroplaxes' in series, and each electroplax consists of two transverse membranes also in series, so that the whole organ is built up of a large number of parallel membranes all in series. Keynes and Martins-Ferreira (1953) inserted a micro-electrode into an electroplax and found that the interior was about 80 mV negative with respect to the interstitial fluid. Thus, there is normally no potential difference across the whole organ since the potential across alternate membranes is equal and opposite. (These investigators also found that one membrane of the electroplax, i.e. every other membrane of the organ, is excitable so that if all the excitable membranes are excited simultaneously a large potential difference will build up across the organ.)

The origin of the resting potential in vertebrate muscle seems to be fairly well understood. Boyle and Conway (1941) have shown that the concentrations of K, Na and Cl in the frog muscle fibre are 126·0, 15·0 and 1·0 mM respectively while in the interstitial fluid they are 2·5, 104 and 74·0 mM. The membrane potential of almost 100 mV measured by Graham and Gerard (1946) may be calculated from the Nernst equation for either Cl or K, thus suggesting that the potential is a Donnan potential brought about by the existence of impermeable ions in the fibre. Experiments involving the measurement of the effect of external ionic concentrations on the membrane potential have

confirmed this. Tracer experiments with radioactive ions (Levi and Ussing, 1948; Harris, 1953) have shown that Cl and K are permeable to the membrane but that Na is only slightly permeable; Hodgkin and Horowicz (1960) have studied the effect of sudden changes in K and Cl concentration on the membrane potential of frog muscle and from the potential transients, observed during the resulting non-equilibrium period, they computed the Cl conductance as being 700 μmho cm^{-2} and the K conductance as being smaller than the Cl conductance by about a factor of ten. However, the fact that the membrane has shown to be permeable to Na raises the question as to why the potential does not gradually run down as the internal K is exchanged for external Na, and so it is necessary to postulate that Na is excreted metabolically by a Na extrusion pump.

The situation in nerve is similar to that in muscle; the resting potential of giant axons is near 60 mV and similar ionic concentrations exist in the interior of axons and in the interstitial fluid. The inside is high in K and low in Na, while the opposite is the case outside. Hodgkin and Keynes (1953) have shown that the effect of changing the external K concentration may be predicted by the Nernst equation except at low values of the resting potential; ~~recently~~ changing the internal K concentration of giant axons (Baker, Hodgkin and Shaw, 1962) has produced exactly the same effect. Cl has been found to have a negligible effect on the potential. Flux experiments indicate that the membrane is permeable to K, so that the membrane potential is determined by the internal and external K concentrations, but, as in muscle, they also reveal

that the membrane is permeable to Na, so that again it is necessary to postulate some mechanism which is responsible for driving Na out of the cell.

The origin of the resting potential in large plant cells is not well understood. These cells are more complicated to deal with because as well as the normal protoplasm found in the interior of all cells they possess a large central vacuole, which is just a salt solution. MacRobbie (1962) has found that the concentrations of K, Na and Cl in the vacuole of Nitella translucens were 78, 37 and 151 mM, while the corresponding external concentrations were 0·1, 1·0 and 1·3 mM. Flux experiments have revealed that all ions are permeable, and since the potential of the vacuole of Nitella translucens is 121 mV (Spanswick and Williams, 1964) it would appear that Cl is well out of equilibrium, for the effect of both the Cl concentration gradient and the electric field would be to drive Cl out of the cell. Thus in order to account for the high concentration of Cl in the vacuole of these cells, MacRobbie has postulated that Cl is transported into the cell by means of a metabolically driven ion pump.

A considerable amount of information is available about potentials across frog skin. Since the frog lives in fresh water it tends to lose salts through its skin; in order to counteract this tendency, frog skin is equipped with a mechanism, driven metabolically, for transporting NaCl from the environment into its extracellular fluid. When the skin separates two identical solutions whose salt concentrations are similar to those of mammalian extracellular fluid, a

potential difference of some 50 mV may be observed. This potential is the sum of two potentials occurring within the skin, and the exact sites of these potentials have been located. The skin comprises a layer of connective tissue on the inside and a layer on the outside consisting of two or three layers of tightly packed cells. It is across the innermost layer of these tightly packed cells that the potential difference occurs, about half of it across the inside membranes and the rest across the outside membranes of this layer of cells (Engbaek and Hoshiko, 1957; Scheer and Mumbach, 1960). Thus the skin may be regarded as a composite membrane consisting of two membranes in series. Flux measurements have shown that Cl and Na are permeable to the skin, and Koefoed-Johnsen and Ussing (1958), having reduced the skin Cl conductance to zero by treating it with copper, found that the effect of changes in outside Na concentration on the potential could be exactly predicted by the Nernst equation while the same was true for the effect of the inside K concentration. At present, it is thought that the outside membrane simply allows NaCl to diffuse into the compartment between the two membranes, while the inside membrane has a high permeability to K and Cl and a low permeability to Na. Na is transported across this inside membrane by means of a non-electrogenic metabolic mechanism, the removal of a Na ion from the compartment between the two membranes being linked to the entry of a K ion from the inside solution.

As has been explained in the previous section, the resistance of a membrane is a measure of the permeability of ions to it;

Further, when current is passed across a membrane the potential builds up exponentially from the moment the current is switched on, finally reaching a steady level; this happens because the capacitance associated with the membrane resistance requires to be charged. All resistance elements have an associated capacitance, and for a resistance of given cross-section the capacitance is inversely proportional to its length. Thus it may be safely predicted that since biological membranes are extremely thin, they should possess an extremely large capacitance. In fact, it has been found that biological membrane capacitances are very nearly 1·0 μF cm^{-2}, whether the membrane belongs to the giant plant cell Nitella, a nerve fibre, or a muscle fibre. This probably reflects the fact that membrane thickness does not vary much from species to species, for it is unlikely that the dielectric constant varies from one lipid to another. On the other hand the membrane resistance varies considerably depending on the species. The value is 21 KΩ cm^2 in Nitella translucens, 8 KΩ cm^2 in crab nerve, 0·7 KΩ cm^2 in squid giant axon and 1·5 KΩ cm^2 in frog muscle. This probably reflects the physiological importance of the membrane resistance in controlling the various ion fluxes through the membrane, since requirements will vary from membrane to membrane. The membrane resistance may be calculated from the ion flux measurements and compared with the measured resistance, but in all cases where this has been done (e.g. Williams, Johnston and Dainty, 1964) the calculated resistance is about a factor of ten times the measured resistance.

1.33 The Excitability of Biological Membranes

It has been mentioned that a potential of some 100 mV exists across the plasma membranes of living cells, the inside being negative with respect to the outside. When this potential is reduced by about 15 mV or more by means of a brief pulse of (depolarising) current, the potential can fall very quickly to zero, even though the 'stimulating' current has been switched off, and then return more slowly to the resting value. This phenomenon is known as the action potential. At the peak of the transient potential the membrane resistance has dropped by about a factor of ten, the membrane capacitance remaining constant. This change in potential occurs locally across the membrane; thus during activity, current will flow from the surrounding parts of the membrane where the resting potential is normal to the part which is excited, and this current will in turn cause the neighbouring regions to become excited, and so on. A wave of excitation will then travel along the membrane with a characteristic speed. This speed is equal to the length of the excited region at any instant times the reciprocal of the duration of the action potential at any point on the membrane. It varies from a few centimeters per second in Nitella to 100 $m.sec^{-1}$ in fast nerves; in squid axon it is 25 $m.sec^{-1}$ (Taylor, 1942). The duration of the action potential is of the order of a millisecond in nerves, and in Nitella it is about 5 seconds (Findlay, 1959).

It is nowadays considered that the action potential is the consequence of the fact that the membrane can allow a transient increase in its conductance of one or more ions; this would

produce a transient potential and resistance change. In those nerves where it has been possible to make the measurements, Na and K conductances have been found to increase during the action potential (Hodgkin and Katz,1949). The same is true of muscle, while in Nitella Cl is thought to be involved. The elucidation of the mechanism by which changes in membrane conductances are brought about remains to be accomplished.

C H A P T E R II

THE RESTING STATE.

2.1 INTRODUCTION

There are certain advantages to be gained from using giant algal cells in the study of the resting state of biological membranes. They are comparatively large, hardy, in plentiful supply at all times and are rarely damaged by the careful insertion of electrodes into their interior. The main disadvantages are that the cells have a vacuole which is bordered by a membrane and that the cytoplasmic membrane surrounding the cell is itself surrounded by a cell wall.

In recent years, with the advent of modern micro-electrode techniques and the availability of radioactive isotopes a great deal of work has been done on membrane resting states in various species of giant algae. Membrane potentials have been measured by Walker (1955), Oda (1956), Kishimoto (1959) and by Spanswick and Williams (1964, 1965). Resistance measurements have been made by Walker (1957, 1960), Kishimoto (1964), Williams, Johnston and Dainty (1964), Findlay and Hope (1964) and by Hope (1965). Ion concentrations in these cells have been measured by MacRobbie and Dainty (1958), Hope and Walker (1960), Oda (1961) and by Spanswick and Williams (1964). Ion fluxes have been measured by MacRobbie and Dainty (1958), Diamond and Solomon (1958), Hope and Walker (1960), MacRobbie (1962), Hope (1963) and by Spanswick and Williams (1965). The effect of external ionic concentrations on the resting potential has been investigated by Kishimoto (1959), Oda (1961), Hope and Walker (1961) and by Spanswick, Stolarek and Williams (in press). The ionic properties of the cell wall

has been studied by Dainty and Hope (1959), Dainty, Hope and Denby (1960) and by Spanswick (thesis). The action potential in the Characeae has also been investigated and this work will be referred to in Section 2.2.

The general picture that has emerged from all this work is somewhat complicated. The cell may be regarded as being composed of three compartments; these are the vacuole, the cytoplasm and the cell wall. The cell wall acts as a cation exchange resin selective to Ca. The bulk of the electrical potential drop between vacuole and the bathing medium is found to occur between the cytoplasm and the bathing medium. The resistance of the vacuolar membrane is about ten times less than that of the cytoplasmic membrane; from this it is to be expected that ion fluxes across the vacuolar membrane should be about ten times smaller than those across the cytoplasmic membrane, and they have been found to be even less. However, in all cases where comparison is possible there is a discrepancy between the measured fluxes and the electrical resistance; the fluxes are too small by about an order of magnitude. Chloride is out of equilibrium across the cytoplasmic membrane and in fact it has been proposed that the action potential is caused by a transient increase in the membrane chloride conductance. Sodium and potassium are also out of equilibrium across this membrane and thus in order to account for the observed phenomenon of flux equilibrium across the cytoplasmic membrane, it has been proposed that in addition to the normal supply of free energy available to power the transport of these ions there is a free energy

supply made available by the cell's metabolism. This metabolic supply of free energy and the mechanism whereby it is utilised is often referred to as an "ion pump".

Since membrane potentials are caused by the existence of free energy differences between one side of a membrane and the other it should be possible to liberate some of the free energy responsible for the resting potential. An electrical experiment in which this is done is described in this chapter. It is also possible in theory to deduce the ionic transport numbers across a membrane without having recourse to flux measurements; it is only necessary to record the effects of different external ionic concentrations on the membrane potential. The method is used in this chapter with some measure of success.

In most of this work the cell wall, cytoplasmic membrane and vacuolar membrane are lumped together and regarded as a single membrane; this simplification is experimentally very convenient. The membrane or resting potential is thus the potential difference between the vacuole and the external solution and this is not very different from that between the cytoplasm and the external solution. Also the resistance of this composite membrane is very nearly the same as that of the cytoplasmic membrane and the effect of external solution changes will in general be due to the cell wall and the cytoplasmic membrane.

Thus by studying this composite membrane it should be possible to obtain considerable information about its components.

2.2 THE RESTING POTENTIAL AS A SOURCE OF ELECTRIC POWER.

2.21 Introduction

Measurements of the resting potential and the internal Cl concentrations in Nitella translucens have shown that Cl is out of equilibrium across the (composite) membrane by about 0·2 electron-volts (Spanswick and Williams 1964, MacRobbie 1962). It has also been shown by Spanswick and Williams (1964) that Na and K are also out of equilibrium, but to a lesser degree. In addition it has been proposed by Gaffey and Mullins (1958), by Mullins (1962) and by Findlay and Hope (1964) that the action potential in the Characeae is due to a transient increase in membrane Cl conductance; this would allow an outward flow of Cl ions which would drive the membrane potential close to the equilibrium potential as given by the Nernst equation.

The fact that Cl is one of the few ions for which reversible electrodes exist makes it possible to study the ion directly by electrical means in Nitella. When the membrane potential is short-circuited by means of Ag/AgCl electrodes, the E.M.F. generated in the circuit is equal to the sum of the membrane potential and the Cl equilibrium potential. From a measurement of the E.M.F. the Cl concentration in the cell may be obtained. Also the current flowing will polarise the membrane and from a study of this effect the electrical resistance and space constant of the membrane may be determined. It is found that the values obtained by this method for the resistance and space constant are in agreement with those obtained by

other workers while values of Cl concentration obtained are in complete disagreement with those of other workers obtained by chemical analysis.

2.22 Methods

The Electrical System.

The wiring diagram is shown in Fig. 2a. The potential difference $\overset{V}{\wedge}$ produced between an Ag/AgCl wire electrode C inside the cell and an Ag/AgCl gauze electrode B outside the cell was measured by the electrometer A. This potential difference could be shorted through R_x by closing the switch S, causing the potential being recorded at A to be proportional to the short-circuit current. R_x could be varied from zero to 1 MΩ. The potential across the membrane was recorded by the conventional microelectrodes M_1 and M_2 inserted at distances x_1 and x_2 from C. Strictly, when current is flowing across the membrane, the potential across a particular part of the membrane should be measured by two electrodes as close as possible to the membrane. If this is not done then serious error will result when the bathing solution is very resistive or the dimensions of the bath very small. However, it was found in practice that in this particular experiment, positioning of M_3 up to 3 cm. from each internal micro-electrode made no difference at all to the reading at A. This may have been due to the large external current electrode. Thus for convenience the external electrode M_3 was sited as in Fig. 2a.

The Electrodes

The internal Ag/AgCl electrodes were made from sections of silver wire about 10 cm. long and about 0·2 mm. in diameter.

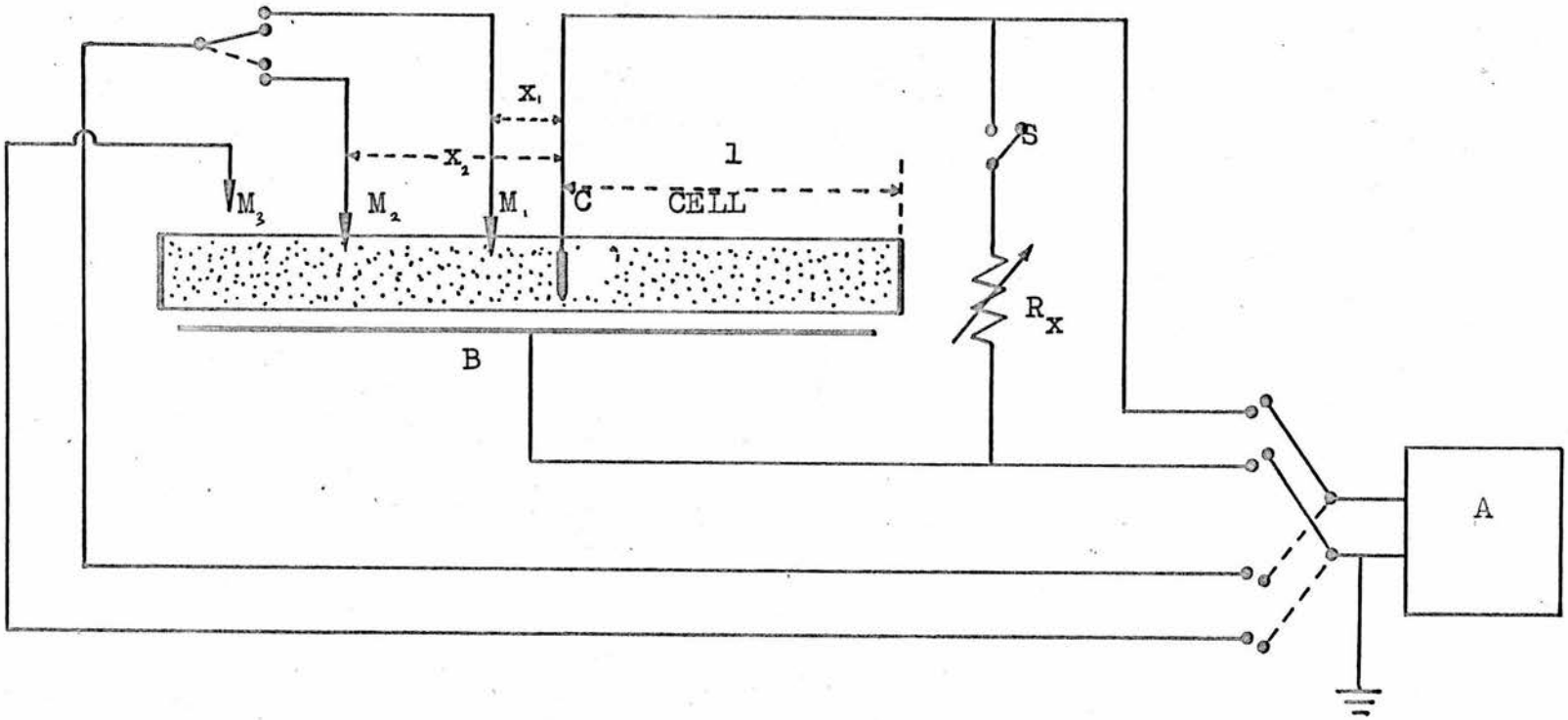


Fig. 2a The electrical arrangement for short-circuiting the membrane potential. M_1, M_2 and M_3 are the potential recording electrodes, and C and B are the current electrodes.

The wire was brought to a fine tip at one end by being electrolytically etched in dilute nitric acid. The tip diameter was between 10 and 30 microns. This tip could be coated with AgCl by using standard methods; however, it was found that the simplest method of coating the required section of the tip was by the insertion of the pointed wire into a Nitella cell and connecting it to an external Ag/AgCl electrode. In a few minutes a brownish black deposit appeared on that part of the electrode inside the cell. The deposit has been taken to be AgCl (see Ives and Janz, 1965). This method is desirable because it ensures that only that part of an electrode which is inside a typical cell possesses a coating of AgCl.

It was found that potentials and short-circuit currents were the same whether the shank of the Ag/AgCl electrode was insulated or not. This was most likely due to the fact that an uninsulated shank in this experiment cannot cause an unwanted short-circuit because the potentials involved are not large enough to cause the evolution of the necessary hydrogen gas at the shank.

The external Ag/AgCl electrode was a strip of silver gauze 1 mm. wide by 10 cm. long, coated with AgCl. The strip was positioned parallel to the cell and as close to it as possible. This gauze was coated with AgCl in a solution of 10 mM NaCl with a current of 10 μA. A different piece of gauze was used with every experiment. The precaution of testing the Ag/AgCl electrode system for tip potentials before experiments was always taken; a few electrodes had to be discarded for this reason for otherwise their tip potentials would have

contributed to the shorting E.M.F.

The micro-electrodes inserted into the cell and used for potential recording were made by attaching micro-pipettes filled with 3N KCl to the tips of commercial calomel electrodes by means of a rubber tube 1 cm. long and filled with 3N KCl. The micro-pipettes were made from sections of pyrex glass tubing of 1 mm. internal bore and pulled out to a fine tip using a micro-forge. The tip diameters were between 1 and 10 microns. Filling the pipettes with 3N KCl was accomplished by repeated boiling in 3N KCl. The resistance of these electrodes was about 5 MΩ. The external potential recording electrode was a calomel saltbridge electrode.

Cable Theory

Williams, Johnston and Dainty (1964) have analysed the cable properties of a Nitella cell. They have shown that if a current I is injected at the center of the cell then the potential change at a distance x from the current electrode due to current flowing across the membrane is given by:

$$\Delta E = I \frac{\lambda}{2}(r_o + r_i) \frac{\cosh \frac{l-x}{\lambda}}{\sinh \frac{l}{\lambda}} \qquad (2.1)$$

where λ is the space constant which is a measure of the extent of the spread of the current along the cell, $(r_o + r_i)$ is the sum of the resistance per unit length of cell interior and the resistance per unit length of external solution, and where $2l$ is the length of the cell. When x=0 the potential change is given by:

$$\Delta E = I \frac{\lambda}{2}(r_o + r_i) \frac{\cosh \frac{l}{\lambda}}{\sinh \frac{l}{\lambda}} \qquad (2.2)$$

so that the quantity $\frac{\lambda}{2}(r_o+r_i)\frac{\cosh l/\lambda}{\sinh l/\lambda}$ must be the effective resistance offered by the cell to current injected at the

centre of the cell.

If we now consider the circuit in Fig. 2a, then if V_s is the short-circuit E.M.F. the total current flowing across the membrane may be deduced from:

$$V_s = I \left\{ R_x + R_s + \frac{\lambda}{2}(r_o + r_i) \frac{\cosh l/\lambda}{\sinh l/\lambda} \right\} \qquad (2.3)$$

where R_s is the resistance of the electrodes. Since the change in membrane potential due to this current is at a distance x from the current electrode given by equation (2.1), then if I is eliminated from equations (2.1) and (2.3) we have:

$$R_x = (\Delta E)^{-1} \left\{ V_s \frac{\lambda}{2}(r_o + r_i) \frac{\cosh \frac{l-x}{\lambda}}{\sinh l/\lambda} \right\} - R_s - \frac{\lambda}{2}(r_o + r_i) \frac{\cosh l/\lambda}{\sinh l/\lambda} \qquad (2.4)$$

Rearranging equation (2.3) gives:

$$R_x = V_s I^{-1} - R_s - \frac{\lambda}{2}(r_o + r_i) \frac{\cosh l/\lambda}{\sinh l/\lambda} \qquad (2.5)$$

In this experiment equations (2.4) and (2.5) are used; R_x is plotted against $(\Delta E_1)^{-1}$ and $(\Delta E_2)^{-1}$ where these correspond to x_1 and x_2; R_x is also plotted against I^{-1}. When it is possible to neglect cable properties equations (2.4) and (2.5) reduce to:

$$R_x = (\Delta E)^{-1} V_s R'_m - R_s - R_m' \qquad (2.6)$$

$$R_x = V_s I^{-1} - R_s - R'_m \qquad (2.7)$$

and these equations are used in Section 2.3.

The External Solution

The composition of the external solution was 0·1 mM KCl, 1·0 mM NaCl and 0·1 mM CaCl. This solution is referred to as APW (artificial pond water).

2.23 Results

A typical plot of R_x against $(\Delta E_1)^{-1}$ and against $(\Delta E_2)^{-1}$ is shown in Fig. 2b. (ΔE_1) and (ΔE_2) are the membrane potential changes at x_1 and x_2. The corresponding plot of R_x against I^{-1} is shown in Fig. 2c. From the slopes of the graphs R_m, $(r_o + r_o)$ and λ

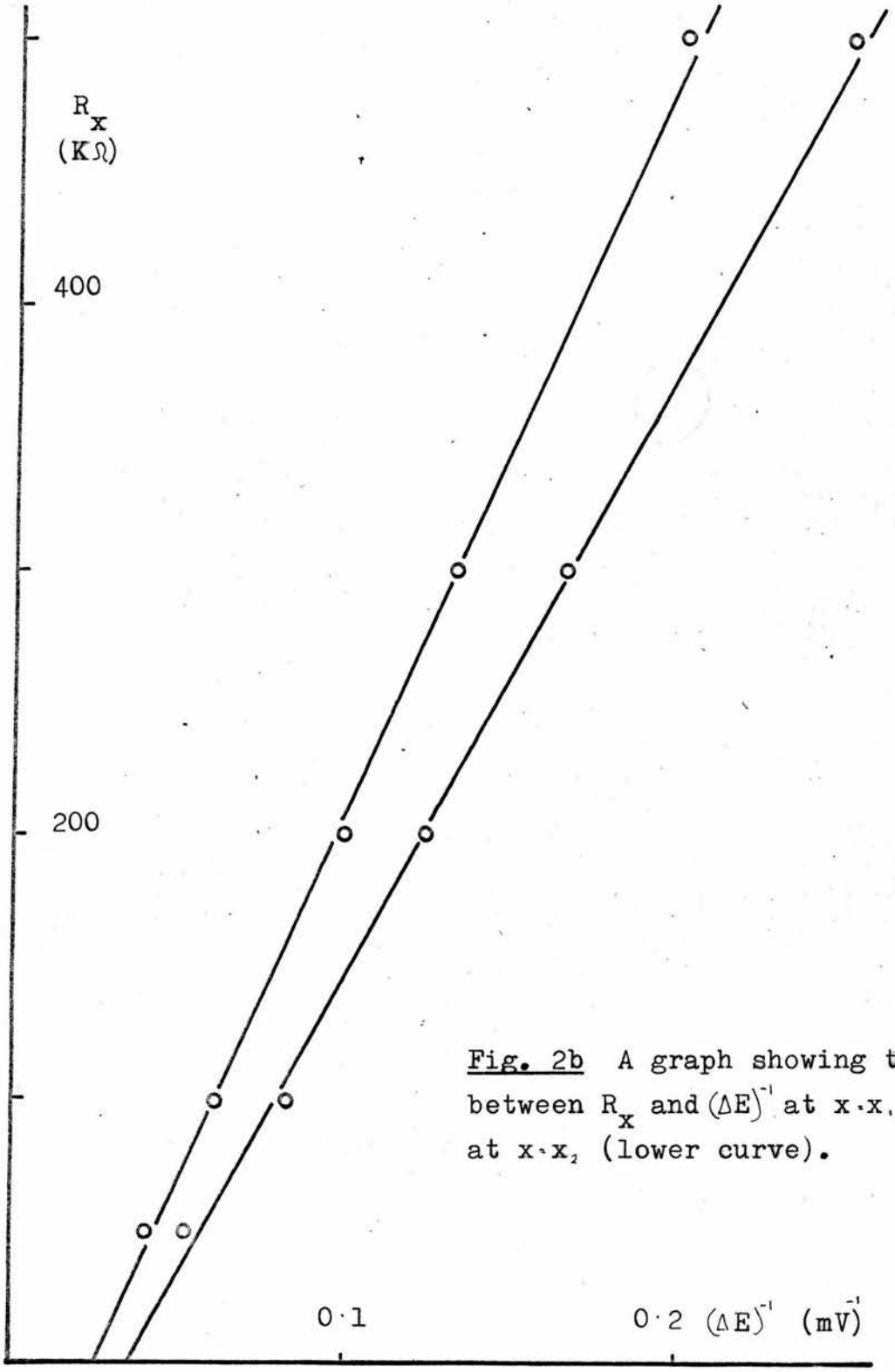


Fig. 2b A graph showing the linear relation between R_x and $(\Delta E)^{-1}$ at $x \cdot x_1$ (upper curve) and at $x \cdot x_2$ (lower curve).

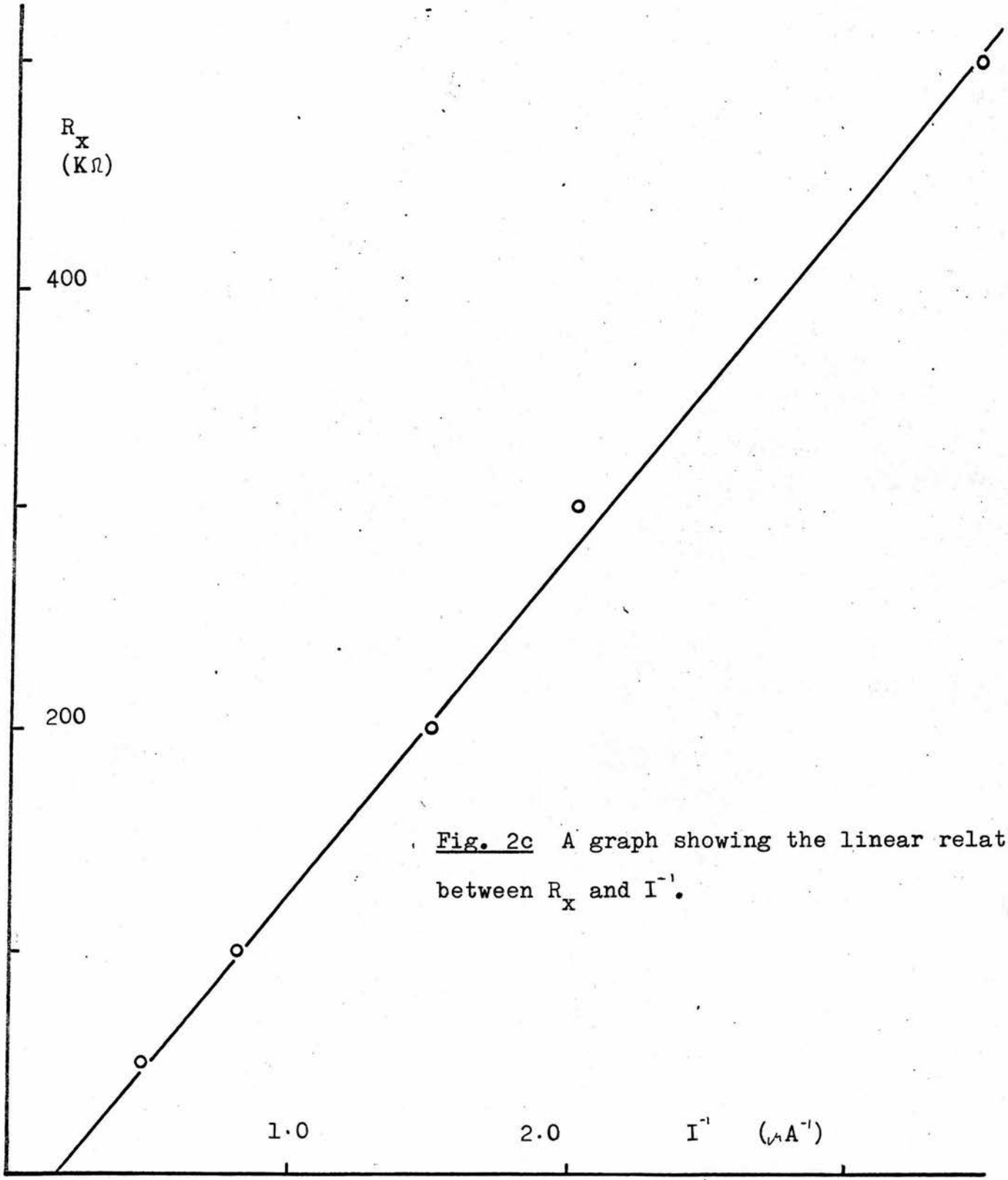


Fig. 2c A graph showing the linear relation between R_x and I^{-1}.

Table 2a. RESULTS OF MEMBRANE RESISTANCE MEASUREMENTS.

Exp.	ℓ (cm.)	λ (cm.)	R_m (KΩcm^2.)
1	3·0	2·0	20·7
2	3·0	2·0	18·6
3	3·0	2·2	10·8
4	4·0	3·5	13·8
5	4·2	3·1	28·3
6	4·5	5·2	26·9
7	4·0	4·0	36·5
8	4·7	2·9	41·8
9	4·0	1·4	10·2
10	4·5	4·1	40.8
Mean		3·0	25·5

Table 2b. INTERNAL CHLORIDE CONCENTRATION RESULTS.

Exp.	E (mV)	V (mV)	C_i (mM)
1	100	169	20·2
2	102	175	29·7
3	110	208	52·8
4	67	162	56·3
5	104	198	54·3
6	95	188	52·2
7	105	186	32·4
8	89	164	25·5
9	120	184	16·5
10	114	205	52·6
Mean	101	184	39 2

may be calculated. The results of experiments on ten cells are summarised in Table 2a. The mean value of the resistance was 25·5 KΩ cm^2 and the mean value of the space constant was 3·0 cm. The errors are between 10% and 15% for the resistance and space constant measurements.

The internal Chloride concentration was calculated from the equation:

$$V - E = \frac{RT}{F} \ln \frac{C_i}{C_o} \qquad (2.8)$$

where C_i and C_o are the internal and external concentrations of Chloride. Table 2b shows the measured values of E and V and the calculated values of C_i. The mean value of C_i was 39·2 mM. V_s was found to be less than V; this was probably due to electrode polarisation.

2.24 Discussion

The Resting Resistance and Space Constant.

The values obtained in this experiment for space constant and resistance are in reasonable agreement with those obtained by Williams, Johnston and Dainty (1964). These workers obtained values of 2·6 cm. and 21 4 KΩcm^2 compared with the values of 3·0 cm. and 25 5 KΩ cm^2. obtained by this short-circuit method. It is important to consider what exactly has been measured by this method. It is most probable that the micro-electrodes were in the vacuole while most of the Ag/AgCl wire electrode tip was probably also in the vacuole. Thus it would appear that the resistance measured in this experiment is the sum of the individual resistances of the vacuolar membrane, cytoplasm, cytoplasmic membrane and the cell wall. However, Findlay and Hope (1964) have shown that the resistance of the vacuolar membrane in Chara

australis is about a tenth of that of the cytoplasmic membrane and cell wall, and MacRobbie (1962) has found very high K and Na fluxes across the vacuolar membrane. Thus, since it is not expected that the cell wall or the cytoplasm contribute significantly to the resting resistance then the resistance measured in this experiment must be approximately equal to that of the cytoplasmic membrane. The same kind of reasoning may be applied to the space constant measurements.

Chloride Concentrations

The values of internal chloride concentration varied between 16·5 and 56·3 mM with a mean value of 39·2 mM. There seems to be some disagreement here with the chloride concentrations measured by Spanswick and Williams (1964) using an electrometric titration method. Their results indicate that the chloride concentration in the cytoplasm was 65 mM and 160 mM in the vacuole. The electrochemical method used in this experiment is clearly not to be taken as being accurate, because while it is most probable that the Ag/AgCl electrode was in the vacuole, it may have been partly or even entirely in the cytoplasm; it must also be conceded that without information about the exact location of this electrode it is not possible to be certain of the significance of the results; however, simple electrochemical theory shows that the concentration determined by this method is intermediate between the cytoplasmic and vacuolar concentrations. Thus even allowing for the inherent inaccuracies of the method the results are still in disagreement with those of Spanswick and Williams (1964).

In seeking a reason for this disagreement the electrodes

are obviously suspect. For example if the Ag/AgCl electrode is in contact with an oxidation-reduction system the electrode potential will not be due to chloride alone but also to the transfer of electrons between electrode and solution. It is well known that biological systems contain many redox components and possible other substances capable of poisoning the electrodes. This kind of phenomenon seems to offer the most likely explanation as to why the electrometric titration and electrochemical results differ.

It has already been mentioned that it has been proposed that the action potential in the Characeae is caused by a transient increase in the chloride conductance of the cytoplasmic membrane. It may be shown that for many purposes a pair of Ag/AgCl electrodes positioned across the cell membrane is equivalent to a region of chloride conductance in the cell membrane. From the graphs it may be deduced that using electrodes whose resistance is small compared with membrane resistance the short-circuit current would drive the resting potential some 170 mV more positive; in other words the theoretical limit to the size of the action potential is about 170 mV. Since the observed action potential in the Characeae is usually less than 100 mV, the experiment merely confirms that an increase in cytoplasmic membrane chloride conductance is a possible cause of the action potential, despite the fact that the results also indicate that the internal chloride concentration is effectively lower than that determined by an electrometric titration method.

2.3 TRANSPORT NUMBERS.

2.31 Introduction

As has been discussed earlier a diffusion potential is a complex phenomenon and no simple theory exists which will deal with all its observed aspects. It is an observed fact that electrical potential differences exist between the inside and the outside of many if not all living cells.To determine to what extent these potentials may be explained by the diffusion of ions across the cell membrane is the aim of a vast research effort. The simplest method of approach is to take one of the many equations connecting diffusion potential with ionic concentrations and experimentally determine whether the observed changes in the resting potential due to changes in the external ionic concentrations may be predicted by that equation. The most common equations used for this are the 'Goldman' equation (Goldman, 1943) and the transport number equation (Hodgkin, 1957) much used in nerve physiology.

The Goldman equation is not very satisfactory because of its implicit assumption of a constant electric field across the diffusion region. The transport number equation is much simpler; this is due to the fact that in this equation the membrane potential depends linearly on the ionic transport numbers of the ions in the membrane and that a transport number is a simple electrical concept, while in the Goldman equation the membrane potential depends on the logarithm of the ion 'permeabilities', where the concept of ionic permeability is not electrical in nature and has to be clearly defined in terms of the kind of electrochemical

theory being used (Dainty, 1962). Recently Finkelstein and Mauro (1963) have shown that for a wide variety of situations the transport number equation may be derived formally from basic theory. Both these equations have been applied with considerable success to the membrane potentials of animal cells; on the other hand with plant cell membranes so far the only systematic experiments involving the relationship between the membrane potential and the external ionic concentrations have been performed using the Goldman equation and the results show that the Goldman equation is capable of predicting the observed phenomena only when calcium is absent from the external solution (Hope and Walker, 1961 and Spanswick, Stolarek and Williams, in press).

The experiments described in this section were designed with a view to determining whether or not the transport number equation is capable of explaining the effect of external ionic concentrations on the membrane potential.

2.32 Materials and Methods

Materials

All the cells used were between 4 and 6 cms. in length to avoid cable theory. There were in fact two batches of cells. One batch was immersed in 5·0 mM NaCl and 0·1 mM KCl solution for 24 hours and then stored in a solution of composition 0·1 mM KCl and 1·0 mM NaCl referred to as calcium free APW. The other batch was soaked in 5·0 mM $CaCl_2$ and 0·1 mM $MgCl_2$ solution for 24 hours and then stored in a solution of composition 0·1 mM $MgCl_2$ and 0·1 mM $CaCl_2$ referred to as calcium solution.

The Electrical System

In these experiments it is simply necessary to measure the resting potential. However since it was intended to measure transport numbers it was decided to measure the membrane resistance as well, as this would enable individual ionic conductances to be deduced if required. The circuit used is shown in Fig. 2d. One micro-electrode positioned near the center of the cell was used to record the resting potential. The resistance was measured by a short-circuit method similar to that described in the previous experiment; however since in this case cable properties could be neglected the simpler equations (2.6) and (2.7) were used to calculate the resistance.

Methods

The experiments on the first batch of cells, i.e. those stored in calcium free APW, involved changing the NaCl concentration to a new value for half an hour, reading the resting potential and then reverting to calcium free APW for another half hour and again reading the resting potential. This process was repeated for 4 NaCl concentrations. Then a similar experiment was performed with the KCl concentration. Before these were begun the resting resistance was measured using the method described in the previous section.

The whole sequence of experiments was repeated with the other batch of cells; this time the $MgCl_2$ and the $CaCl_2$ concentrations were varied in a systematic manner. The table gives the Na, K, Ca and Mg concentrations used.

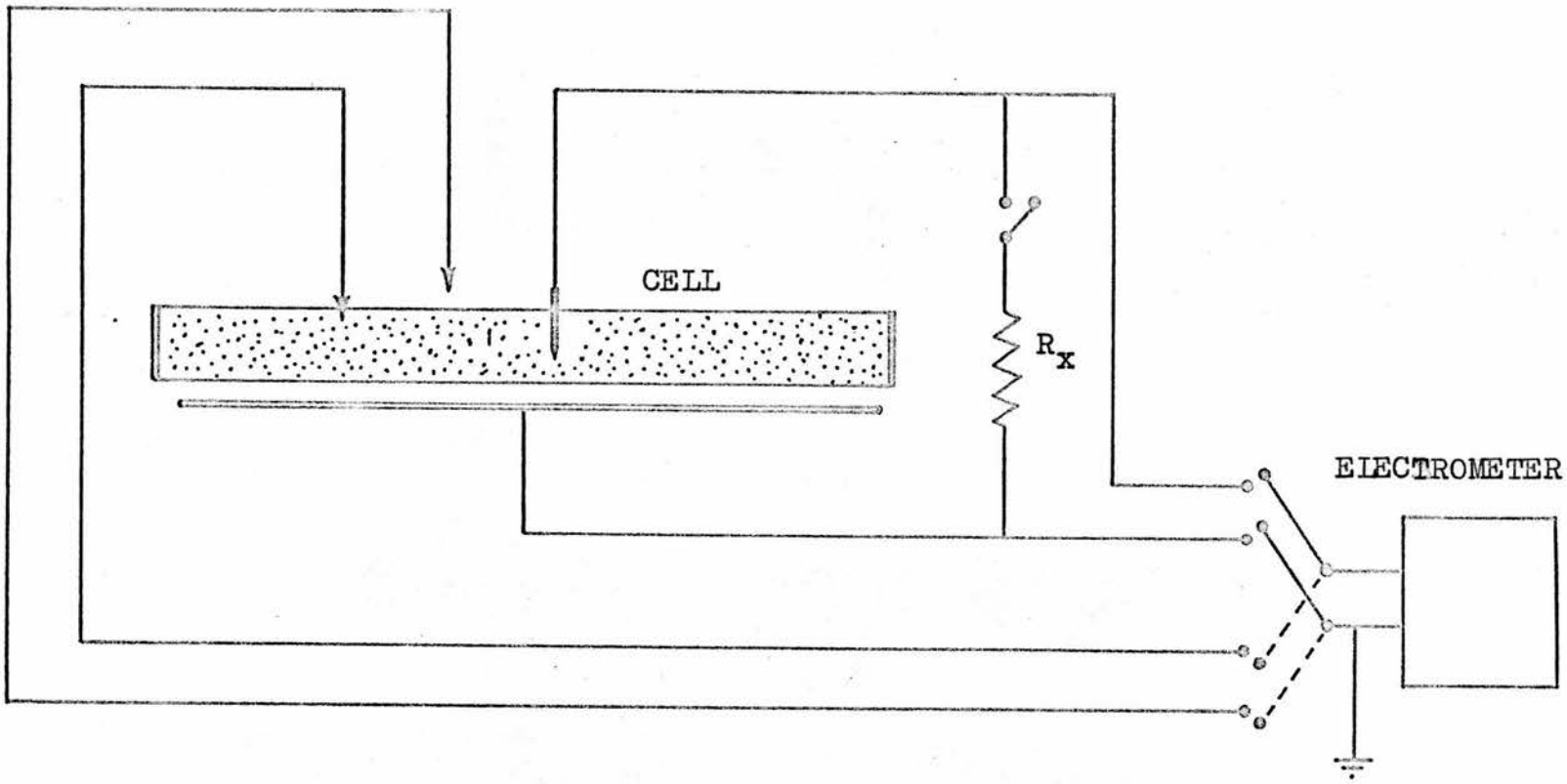


Fig. 2d The electrical system for determining the membrane transport numbers and resistance.

CONCENTRATIONS OF THE VARIABLE IONS IN THE SOLUTION CHANGING EXPERIMENTS.

First Cell Batch		Second Cell Batch	
Exp. 1.	Exp. 2.	Exp. 1.	Exp. 2.
Na (mM)	K (mM)	Ca (mM)	Mg (mM)
0·1	0·1	0·1	0·1
0·3	0·3	0·3	0·3
1·0	1·0	1·0	1·0
4·0	4·0	4·0	4·0
10·0	10·0	10·0	10·0

Table 2c.

2.33 Results

According to the transport number equation the resting potential E_m is described by: $E_m = \sum_n \frac{RT}{Z_n F} T_n \ln \frac{C_n^o}{C_n^i}$ (2.8) where T_n is the transport number of the nth ion and C_n^i and C_n^o are the internal and external concentrations of that ion. Since chloride has not been observed to have any appreciable effect on the resting potential (Spanswick, Stolarek and Williams, in press) and since during the period of the experiment the internal ionic concentrations cannot be expected to change significantly, then for the experiment dealing with Na concentration changes E_m should be described by:

$$E_m = T_{Na} \frac{RT}{F} \ln C_{Na}^o + \text{constant} \qquad (2.9)$$

with similar equations applying to experiments involving K, Ca and Mg concentrations changes.

Graphs were plotted of E_m against $\log_{10} C_{Na}^o$, of E_m against $\log_{10} C_K^o$, of E_m against $\log_{10} C_{Ca}^o$ and of E_m against $\log_{10} C_{Mg}^o$. Typical curves are shown in Figs. 2e, 2f, 2g and

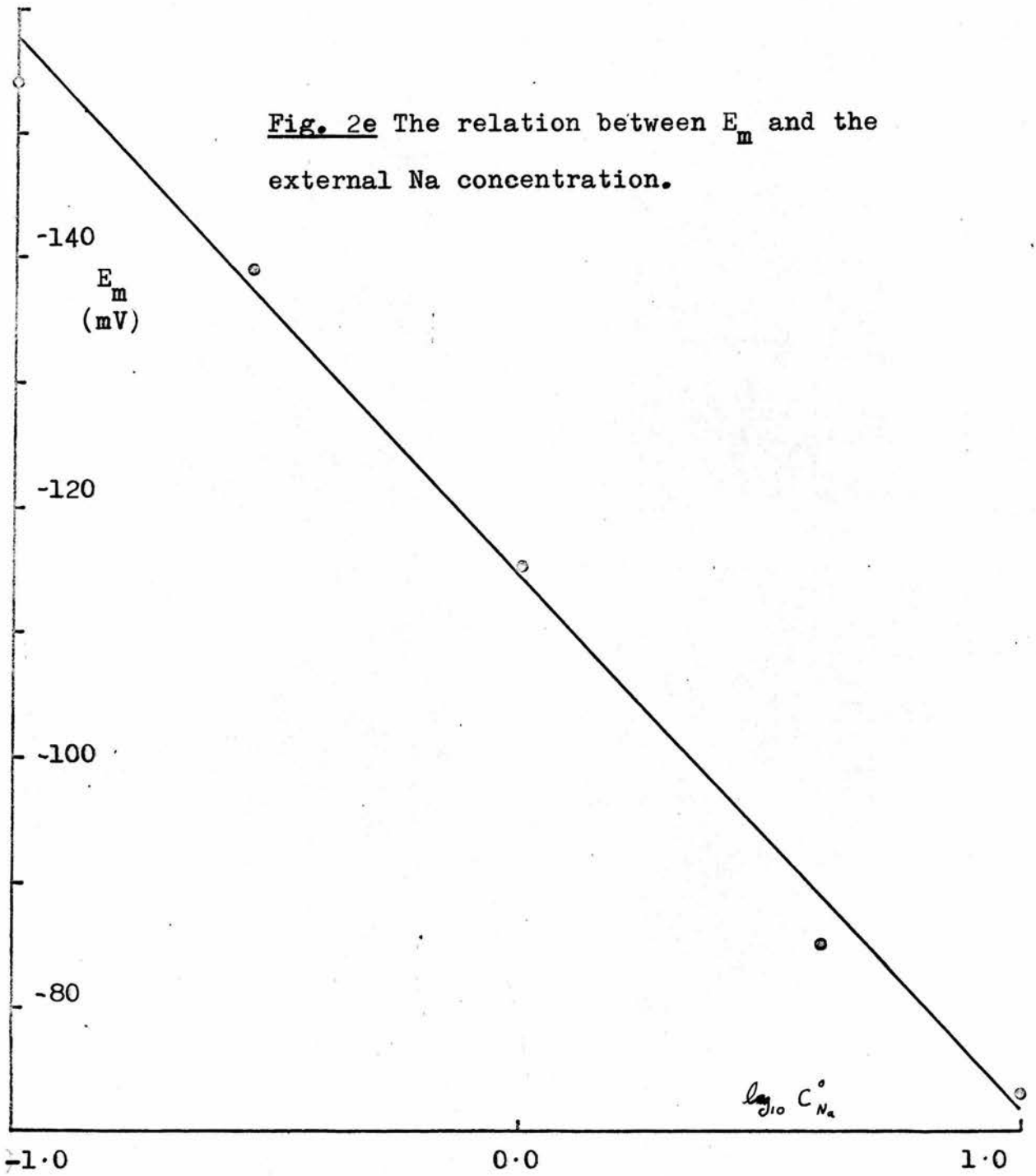


Fig. 2e The relation between E_m and the external Na concentration.

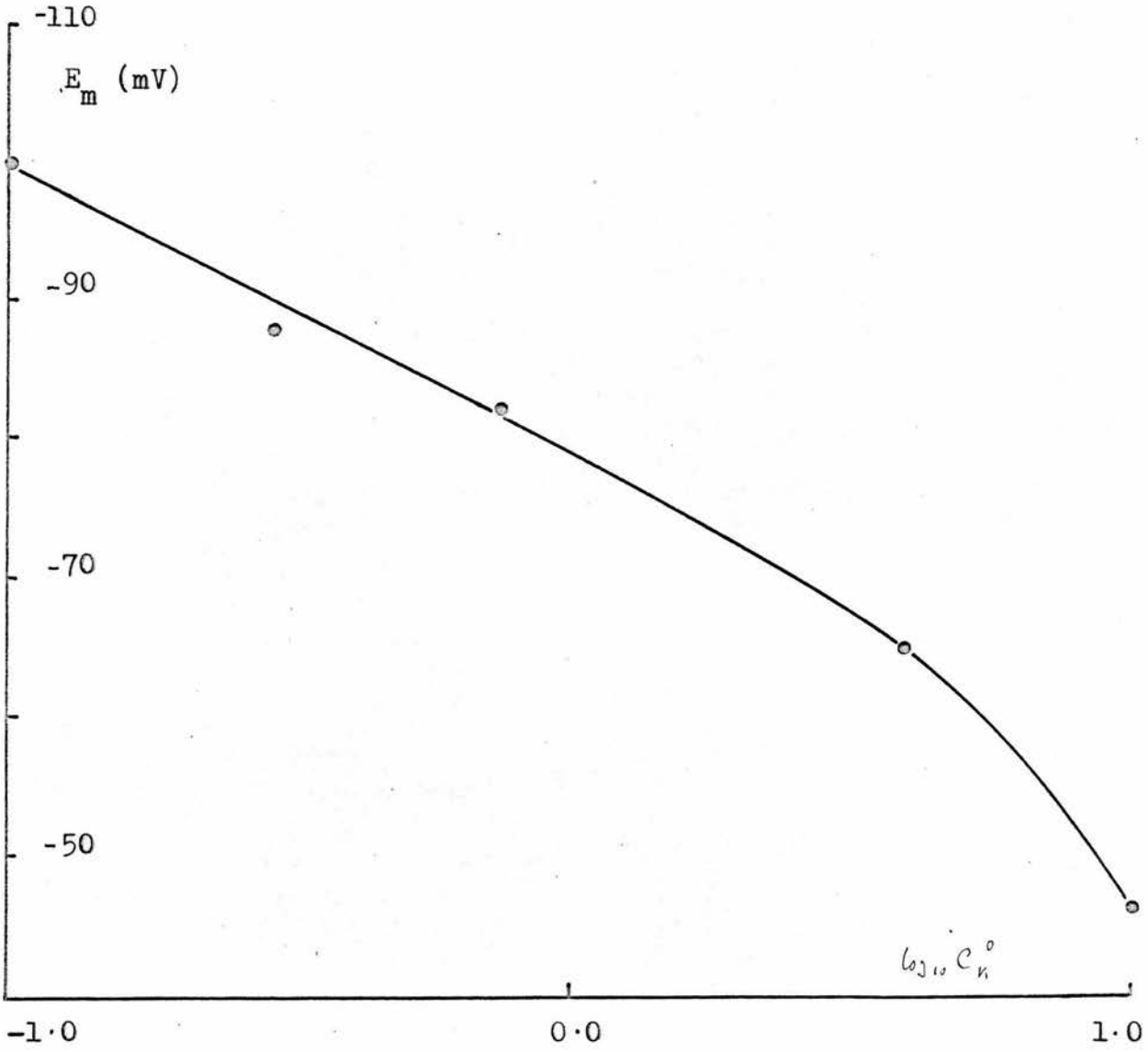


Fig. 2f The relation between E_m and the external K concentration.

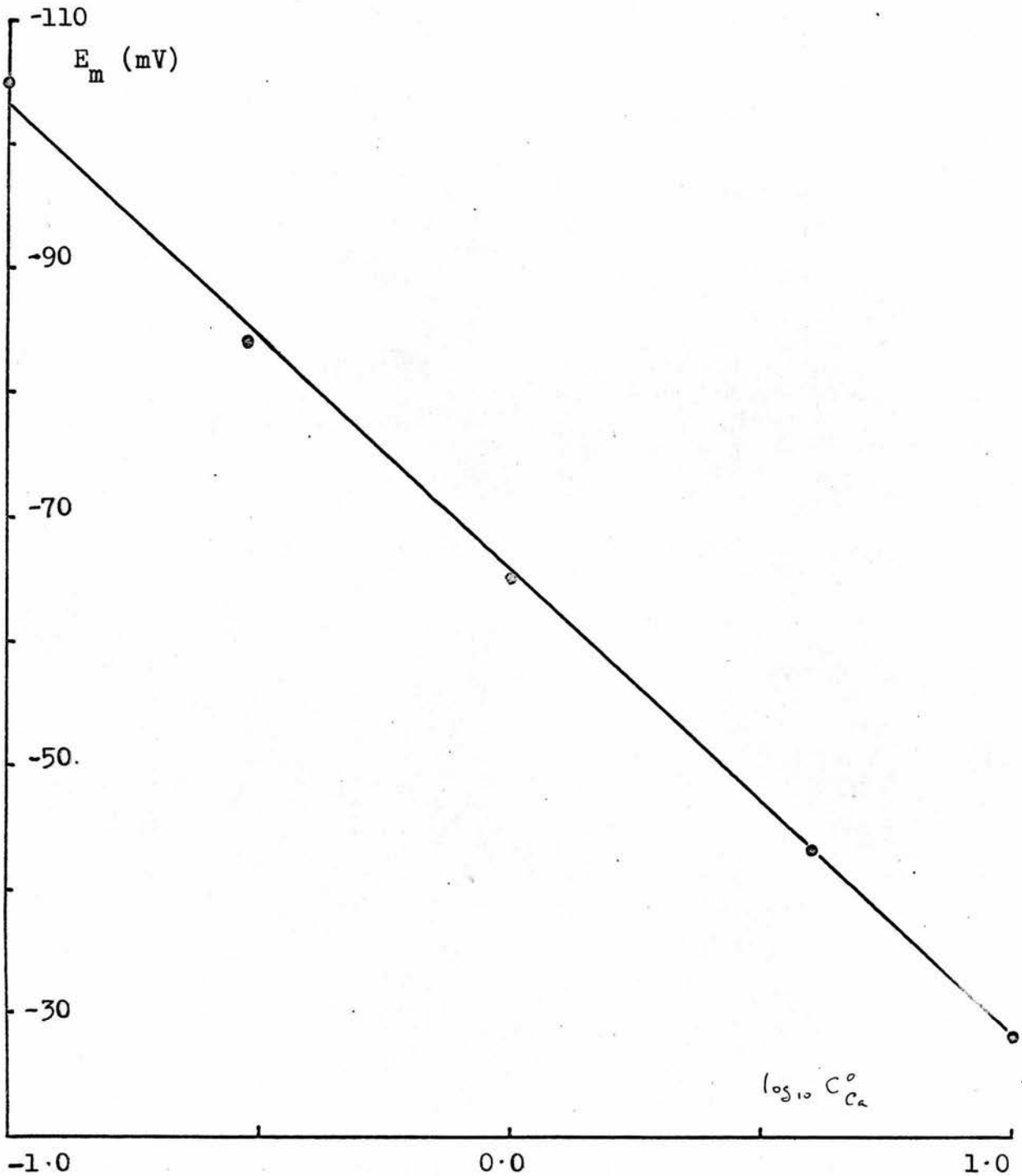


Fig. 2g The relation between E_m and the external Ca concentration.

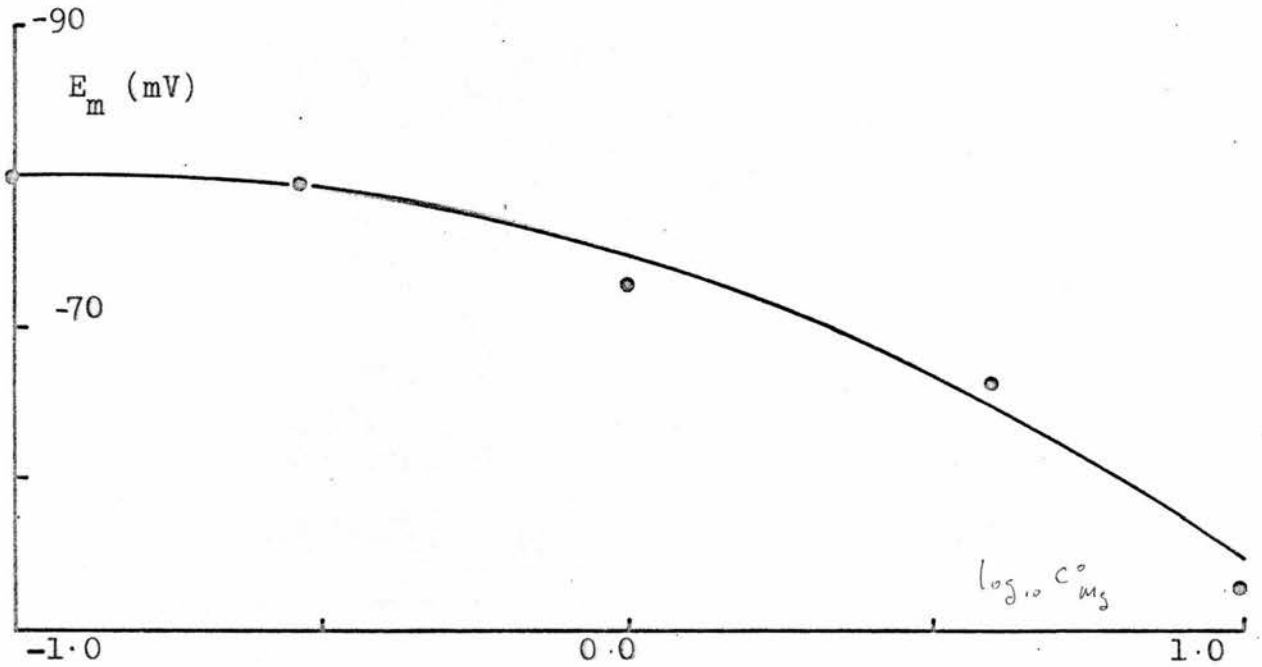


Fig. 2h The relation between E_m and the external Mg concentration.

Table 2d. RESISTANCE AND Na AND K TRANSPORT NUMBERS.

(First Batch in 0·1 mM KCl, 1·0 mM NaCl solution)

Exp.	R_m (Kℓcm².)	T_{Na}	T_K
1	--	0·53	0·40
2	18·7	0·75	0·33
3	13·6	0·75	0·25
4	29·6	0·82	0·33
5	20·6	0·77	0·08
6	14·7	0·77	0·12
7	14.4	0.83	0.08
Mean	18·6	0·75	0·20

Table 2e. RESISTANCE AND Ca AND Mg TRANSPORT NUMBERS.

(Second Batch in 0·1 mM CaCl, 0·1 mM MgCl solution)

Exp.	R_m (Kℓcm².)	T_{Ca}	T_{Mg}
8	16·3	1·03	0
9	27·9	0·73	--
10	27·0	1·00	0·40
11	14·1	1·17	0
12	43·3	1·27	0
13	16·3	0·93	0
14	60·6	0·97	0·23
15	48·2	1·33	0
16	16·6	0·97	0·17
Mean	27·8	1·00	0·10

2h. The transport number of each ion was taken as the slope of the appropriate curve at the point where the ion concentration was that of the reference medium.

It will be noticed that the Na and Ca curves are straight lines while the K and Mg curves are not. This would appear to indicate that the Na and Ca transport numbers are independent of Na and Ca concentrations respectively and that the K and Mg transport numbers increase with increasing K and Mg concentrations respectively; this increase in K and Mg transport numbers must presumably be at the expense of other ionic transport numbers since the algebraic sum of the membrane transport numbers cannot exceed unity.

In order to deduce the membrane resistance graphs of R_x against $(\Delta E)^{-1}$ and of R_x against I^{-1} were plotted; the resistance was calculated from equations (2.6) and (2.7). The resting resistances and the Na and K transport numbers obtained from the first batch of cells are shown in Table 2d. The mean value of the resistance was 18·6 KΩcm^2; the mean values of the Na and K transport numbers were 0·63 and 0·20 respectively. The data obtained from the second batch of cells is shown in Table 2e. The mean value of the resting resistance for this batch was 27·8 KΩcm^2, while the mean value of the Ca and Mg transport numbers were 1·0 and 0·1.

2.34 Discussion

First of all, the observed phenomena in the experiments described in this section will be considered in the light of the phenomena observed by other workers in similar and related experiments.

Spanswick, Stolarek and Williams (in press) have found that for Nitella cells bathed in APW, calcium is the only ion which has a significant effect on the resting potential. The author (see Chapter 111) has also observed this effect in voltage and current clamp experiments. Spanswick and Williams have also found that with cells pretreated with 5·0 mM NaCl both Na and K have a significant effect in Ca free solution. The same phenomena have been observed in the experiments with Na and K described in this section. Hope and Walker (1960) have also observed this effect in Chara. Further the experiments with Ca and Mg described in this section show that of these two fairly similar ions only Ca has a significant effect.

Spanswick (thesis) has found that the cell wall is a cation exchange resin having an indiffusible anion concentration of about 0·7 equiv. l^{-1}. Also in experiments involving the effect of different solutions on the diffusion potential across the isolated cell wall Spanswick (thesis) has found that Ca has the most significant effect.

However flux measurements made by MacRobbie (1962) show that the net passive Na and K fluxes in Nitella are some twenty times larger than the net Ca fluxes measured by Spanswick and Williams (1965).

The fundamental point that seems to have emerged is that if the flux measurements are reliable, then Ca ought to have a negligible effect on the resting potential compared with Na and K; experimentally, the opposite is found to be the case.

Thus the results of solution changing experiments and flux experiments are in conflict and this does not depend on the

kind of electrochemical theory being used; moreover this dilemma does not seem to be appreciated in the literature. A very simple explanation of these phenomena is that the potential difference between the cytoplasm and the bathing medium is due to two diffusion potentials in series, one across the cell wall and the other across the cytoplasmic membrane. It may be simply calculated that the cell wall resistance is small compared with the plasmalemma resistance and thus ion fluxes between the cytoplasm and the bathing medium must be rate limited by the plasmalemma membrane alone. On the other hand the diffusion potential across the cell wall will depend upon the ion concentrations in the external solution; this makes it difficult to decide to what extent the plasmalemma potential also depends on the external solution.

The cell wall is known to behave as an ion exchange resin and Dainty and Hope (1959) and Dainty, Hope and Denby (1960) have shown that the ions in the cell wall obey the Donnan relations and that the cell wall calcium can be completely exchanged for sodium. Spanswick (1964) has estimated that the concentration of indiffusible anions in the bulk of the cell wall (this excludes that volume of the cell wall which is merely an extension of the external medium) is 0·74 equiv. l^{-1}.

It is thus highly probable that the cell wall diffusion potential is a Donnan potential, and since Ca is divalent its concentration in the cell wall will approach that of the indiffusible anions, so that the Cl concentration in the wall will be much less than in the bathing medium. Thus when the external solution is APW, Ca will have a large effect on the

resting potential and Cl will have little effect. This also means that when Ca is exchanged out of the wall by Na and the external solution is Ca free APW then Na will have the largest effect on the membrane potential; however when the external K concentration is greater than the Na concentration then K will have the greatest effect on the potential. Cl would not be expected to have any effect. Thus the changes in potential observed in the experiment where the Na and K concentrations were changed can be qualitatively explained as being cell wall potential changes, and there is no need to invoke plasmalemma potential changes. In this experiment the concentrations of Na and K in the cell wall must also be appreciably changed so that if Na and K are permeable to the plasmalemma then a change in the plasmalemma potential must also occur. However, since MacRobbie (1962) has shown that the fluxes of Na and K are too small to account for the observed membrane resistance, this most probably means that the Na and K membrane conductances are small so that Na and K concentration changes in the cell wall are unlikely to have an appreciable affect on the plasmalemma potential.

The effect of Ca on the resting potential observed in the present experiments is clearly due to the cell wall potential. The small effect of Mg is somewhat unexpected and must mean that the ion cannot penetrate the cell wall easily.

It is concluded that the observed potential changes were all due to the cell wall.

2.4 GENERAL DISCUSSION

It has been demonstrated that a Nitella cell has an accessible store of free energy and that this energy may be released by employing the cell as an electrochemical concentration cell. The measurement of the membrane resistance then reduces to measuring part of the internal resistance of a biological battery. Although considerable use has been made of this effect in this chapter, it is not to be recommended for use as a routine laboratory method for external power supplies are much more flexible. The experiment clearly demonstrates the mechanism by which cellular free energy may be dissipated electrically and that there is a very close parallel in the method by which power is dissipated during the action potential. The solution changing experiments support the hypothesis that the cell wall and the cytoplasmic membrane behave as a composite membrane. Thus solution changing experiments will produce cell wall effects due to the latter's ion exchange properties, while, since the cytoplasmic membrane resistance is greater than the cell wall resistance, in flux experiments and in experiments involving the passage of electric current between the interior and the exterior of the cell, then it is the cytoplasmic membrane which is being studied. Further since the cell wall and the cytoplasmic membrane are in series to what extent the resting potential is caused by either component may only be guessed at. However it is highly probable that changes in the resting potential caused by the action potential are cytoplasmic membrane phenomenon.

In the next chapter, the membrane is studied in the wider context of both its resting and excited states.

C H A P T E R III

THE RESTING AND EXCITED STATES

3.1 INTRODUCTION

3.11 The Action Potential

The action potential in the Characeae was first observed by Osterhout and Hill (1930a) working on a Nitella species. It had been known from the work of Osterhout and Harris (1928) that when a small section of a Nitella cell is poisoned with chloroform, injury currents flow between the injured and uninjured parts of the cell. This was correctly understood to be due to the fact that the electrical potential across the injured portion of the cell membrane was different from that across the uninjured portion. While studying these injury currents Osterhout and Hill (1930a) discovered that if they were made large enough, then potential transients apparently due to the uninjured part of the cell could be observed at frequent intervals. They pointed out that these potential transients were similar to those observed with nerve cells.

It must be remembered that at the time of these observations the standard method of recording electrical activity in nerve cells was to place one recording electrode at the killed end of the cell while the other was placed in contact with the intact portion of the cell. This arrangement of the recording electrodes is called a monophasic lead and the potential transient from a nerve cell recorded with this arrangement is referred to as a monophasic action potential. The monophasic lead was used by Osterhout and Hill and so what they recorded was thus a monophasic action potential. The monophasic lead has been superseded by the development of intracellular electrodes.

Further work by Osterhout and Hill (1930b) on Nitella

demonstrated for the first time the decisive role played by local circuits in the propagation of an action potential; they found that they could reversibly block the propagation of an impulse by disconnecting a salt-bridge which formed part of a local circuit. They were thus able to confirm for Nitella the prediction made by Hermann (1899) that impulse propagation in nerve may be assisted by a local flow of current between the resting and the excited portions of the cell. The same effect was later observed in isolated invertebrate nerve fibers by Hodgkin (1939) and in single nerve fibers of the toad by Tasaki (1939).

In a series of experiments on Nitella, Umrath (1930, 1932a, 1932b, 1934) essentially confirmed the work of Osterhout and Hill. He managed to insert a fine glass capillary filled with a conducting substance into the cell and was able to record the action potential.

In 1938, Cole and Curtis measured the resistance of the membrane of Nitella during excitation and discovered that it was significantly less than the resting value. They used external electrodes and the cell was connected into an alternating current Wheatstone bridge. Cole and Curtis (1939) then repeated the same experiment with squid giant axon and were able to demonstrate that the action potential is accompanied by a pronounced change in membrane resistance. More recently Hodgkin and Huxley (1952a, 1952b) and Hodgkin, Huxley and Katz (1952) measured the resistance of squid axon during excitation by a novel method which involved clamping the membrane potential during excitation by a feedback system

and recording the variation in peak membrane current with membrane potential. The advantage of this method over the converse method of measuring the variation of peak membrane potential with constant membrane current lies in the fact that the peak transient current in the constant voltage method is independent of the membrane capacitance if the experiment is properly designed, while in the constant current method the value of the peak transient potential is affected by the membrane capacitance. The voltage-clamp technique, as the constant voltage technique has come to be called, has proved very successful and has been used to study other excitable cells. In recent years it has been employed on Chara and Nitella by Findlay (1962, 1964) and by Kishimoto (1964). The technique has made it possible to obtain the current-voltage characteristic of an excited membrane. The fact that the curve for these excited membranes is linear may be taken as showing that an excited membrane, just like a resting membrane, behaves like a battery with a definite electromotive force and internal resistance.

The electrochemical nature of the action potential has been the subject of study and speculation for a long time. Bernstein produced the first electrochemical theory of the action potential. According to his theory the resting membrane potential was a Donnan potential with K as the permeable ion and the action potential was due to a nonselective increase in the membrane permeability to ions. The fact that Osterhout (1934) found that the membrane of a Nitella cell behaved like a potassium electrode was evidence in support of the Bernstein theory. However,

Osterhout (1934) postulated that the action potential in Nitella was due to the membrane increasing its permeability to K thus causing the internal K concentration to drop. He was not able to confirm his theory experimentally.

In 1949, Ling and Gerard found that in squid axon, within certain limits, the resting potential was related to the internal and external concentrations of K by the Nernst equation, and this fact was later confirmed by Hodgkin (1951). This finding gave support to the Bernstein theory. Previously, however, Cole and Curtis (1940) found that the amplitude of the action potential exceeded that of the resting potential; according to the Bernstein theory it should be about equal to the resting potential. Then in 1949 Hodgkin and Katz discovered that the amplitude of the action potential in squid axon varies with 58 mV times the logarithm of the concentration in the external solution and they postulated that the membrane potential at the peak of activity is determined by the concentration gradient of the Na ion across the membrane.

Hodgkin and Huxley (1952a, b, c, d) have developed this idea even further. They explain the action potential as being brought about by a specific increase in membrane Na conductance followed by an increase in K conductance, and they have experimentally substantiated their theory by voltage-clamp experiments and flux measurements with radioactive tracers. At present this is the only widely accepted theory of action potential production.

In 1958, Gaffey and Mullins investigated the electrochemical nature of the action potential in Chara globularis. They

determined the concentration of K, Na and Cl in the vacuole of the cell and calculated the equilibrium potentials of each of these ions. They found that K was close to electrochemical equilibrium and that Na was out of equilibrium while Cl was a long way from electrochemical equilibrium. This fact indicated that the Cl ion might be able to cause an action potential by increasing its membrane conductance and thus shifting the resting membrane potential towards the Cl equilibrium potential. They also found, using radioactive tracers, that Cl fluxes increased during the action potential and they concluded that the action potential was caused by an increase in membrane Cl conductance. They also found some evidence that the peak of the action potential depended on the external Cl concentration. Very recently, Hope and Findlay (1964) have studied the Cl fluxes during the action potential in Chara and have confirmed the work of Gaffey and Mullins. Thus it would appear that the electrochemical basis for excitation in large plant cells is different from that for nerve cells.

It has been known for a long time that the strength of stimulφus required to excite a nerve times its duration is approximately a constant. Nernst (1908) formulated an ionic theory of nerve excitation to explain this. He was able to derive the observed strength-duration relation by assuming that excitation took place when the concentration of some ion in or near the membrane reached some critical level.

With the development of intracellular electrodes, it became possible to pass square current pulses through an excitable membrane and so measure directly the relation between the

applied current and the minimum time required to excite the membrane. This type of experiment has been performed on nerve by several workers (e.g. Hodgkin and Rushton, 1946) and on Nitella by Findlay (1959). A threshold depolarisation has been defined as the smallest depolarisation which can decay without causing an action potential. The value found for most nerves is about 15 mV and the value found for Nitella by Findlay is about the same.

The time course of an action potential in a nerve fibre is not affected by the rate at which stimulating shocks are applied provided this rate does not exceed about 10 per second. In fact, the maximum rate at which action potentials of any profile can be produced by a nerve is about 100 per second. Since a nerve action potential lasts about a millisecond, there is a period of up to 10 msecs. following excitation in which it is not possible to excite the fibre. This period is called the refractory period. The same effect occurs in Nitella and in these cells the refractory period can last up to 30 secs. No systematic studies of the refractory period in Nitella have ever been made, although it has been known for a long time that cytoplasmic streaming, which suddenly ceases at the peak of the action potential, does not occur during the refractory period, (see Findlay, 1959).

Weidmann (1951) has shown that in cardiac muscle of the kid, the membrane in its excited state can be induced to make a transition to the resting state by means of a hyperpolarising pulse injected at the peak of the action potential; this appears to be the converse of the process of stimulation, and

in this way he showed that the recovery stage of the action potential and the refractory period could be abolished. The effect was also later observed in toad nodal membrane by Tasaki (1956) but it is not known yet whether it occurs in Nitella.

3.2 VOLTAGE-CLAMP EXPERIMENTS

3.21 Introduction

The voltage-clamp technique was developed by Cole (1949) and by Hodgkin, Huxley and Katz (1949) to study the excitable membrane of the squid giant axon. Basically the technique involves the injection of square voltage pulses across the membrane. These pulses are of variable strength, duration and polarity. Since the injection of square voltage pulses into any electrical system is often difficult, for work on biological membranes special techniques have had to be developed. The current response of an excitable membrane subjected to a square voltage pulse is of fundamental importance and has been studied by many workers.

In general it is found that the current response of such a membrane depends upon the external ionic concentrations. From a study of this effect in squid axon and from studies of ion fluxes, Hodgkin and Huxley (1952) put forward the hypothesis that accompanying the action potential is a transient increase in the membrane conductance of Na, followed by a transient increase in the membrane conductance of K. Since then however, the excitable membranes of other cells, both plant and animal, have been studied under a voltage clamp. So far the animal cells studied have been squid giant axon by Hodgkin and Huxley (1952a, b, c, d), frog node by Dodge and Frankenhaeuser (1958) and lobster giant axon by Julian, Moore and Goldman (1962). The information emerging from these studies lends support to the Hodgkin-Huxley hypothesis. Voltage-clamp studies on excitable plant cell membranes have been made by Kishimoto (1964),

working on a Nitella species and by Findlay (1962, 1964) on a Nitella species and on Chara australis. These experiments have shown that both Nitella and Chara have fairly similar current responses to nerve cells, but similar effects of Na and K have not been observed. In fact, Gaffey and Mullins (1958) and Hope and Findlay (1964) have proposed, on the basis of Cl flux measurements performed during the action potential, that excitation is accompanied by a transient increase in the membrane conductance of Cl. It is thus to be expected that Cl should affect the current response of these giant cells to a square voltage pulse; this has not been found to be the case and the only ion found to affect the response significantly is Ca. The present experiments further confirm this. Frankenhaeuser and Hodgkin (1957) have reported that Ca has a marked effect on the current response of the squid axon membrane and a somewhat similar effect has been reported by Julian, Moore and Goldman (1962) for lobster giant axon.

The present series of experiments were performed on the excitable membrane of the giant plant cell, Nitella translucens, with the object of comparing its current response with those of other excitable membranes and of identifying the charge carriers in the clamp currents. The possible effect of the cell wall on the excitable membranes of plant cells is also considered.

3.22 Methods

The Electrical System.

To illustrate the principles of both the design and the operation of the voltage-clamp system, the electrical analogue in Fig. 3a. will be taken as representing an excitable membrane.

The component values correspond roughly to those encountered in practice. The switch, which is normally open, would, if closed momentarily, initiate the potential transient shown in Fig. 3b. The resistance of this membrane would also momentarily drop to about a tenth of the normal value. Since transient potential and resistance changes appear to be the primary electrical characteristics of excitable membranes, the analogue is thus fairly realistic.

In order to inject square voltage pulses into this system, a switch and a voltage source are the main requirements. The internal resistance of the voltage source must be small compared with about 5 KΩ, which is the value of the membrane resistance with the switch closed. The required circuit is shown in Fig. 3c. It is instructive to study the current response of this system to a square voltage pulse if, during the period of the pulse, the 'membrane' becomes excited due to the closure of the switch; the response is shown in Fig. 3d. The overshoot at the beginning and end of the response is due to the membrane capacitance and the time constant of the effect is given by:

$$\tau = \frac{C_m R_i R_m}{R_i + R_m} \sim C_m R_i \qquad (3.1)$$

so that the effect may be reduced by lowering the resistance of the voltage source.

The important point to note is that the current becomes reversed during excitation and will increase in magnitude under certain circumstances. This also happens in the current response of excitable membranes and may be simply explained. The membrane analogue is resting and excited when the switch is open and

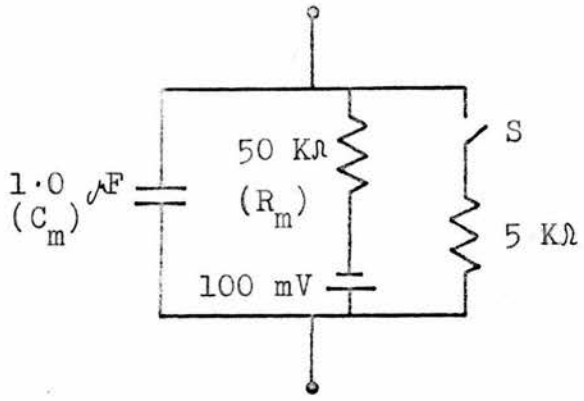


Fig. 3a Membrane analogue.

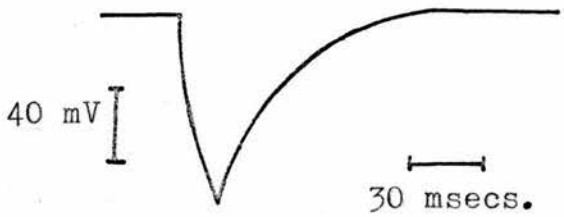


Fig. 3b Excitation caused by closing the switch S.

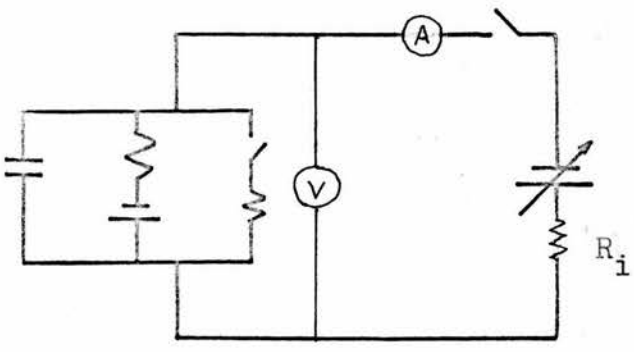


Fig. 3c Simple voltage-clamp.

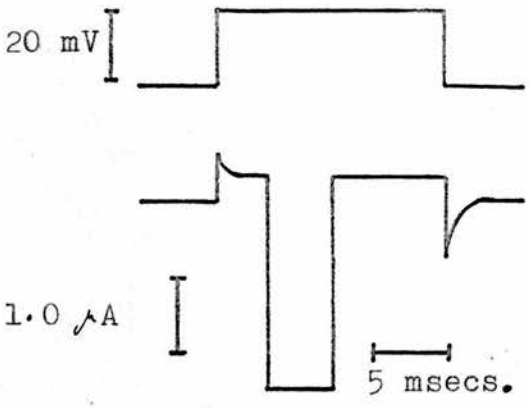


Fig. 3d Current response to square voltage pulse.

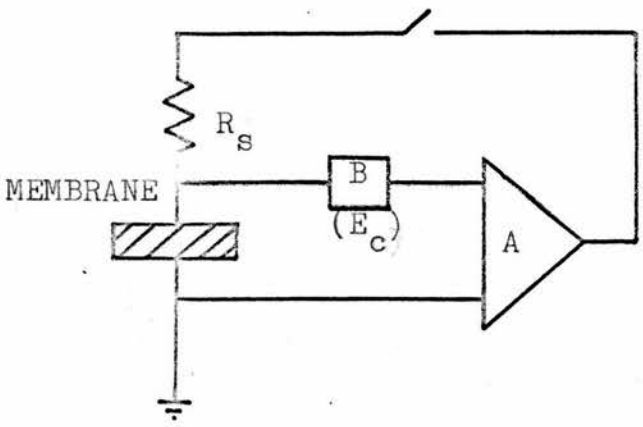


Fig. 3e Basic feedback voltage-clamp system.

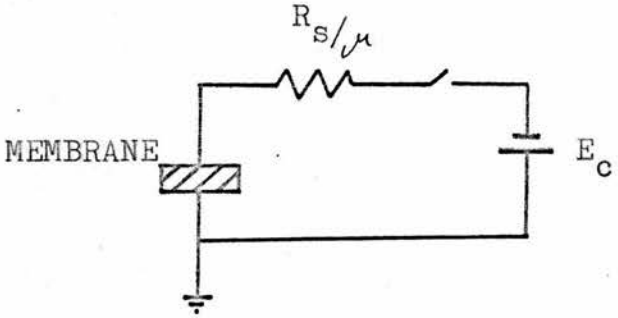


Fig. 3f Basic equivalent circuit of feedback system.

closed respectively. Let us imagine that in both cases the membrane is clamped with the inside 80 mV negative with respect to the outside; in the resting membrane it is clear that there is a net voltage of 20 mV driving an outward current of 0·4 μA through 50 KΩ. When the membrane is excited it generates a potential of about 10 mV with an internal resistance of 5 KΩ; thus, when the membrane is clamped at 80 mV there is a voltage of about 70 mV driving a current of some 14 μA inward through a resistance of about 5 KΩ. So, if the membrane makes a transition from the resting state to the excited state while the voltage is clamped at 80 mV by the external voltage source, then the current becomes reversed and increases in magnitude. In other words it may be said that when a polarising clamp voltage is less than the resting potential then the current is outward and small, but should the membrane become excited then the same polarising clamp voltage can be greater than the excited membrane potential and so the current is now inward and large; all this follows simply from Ohm's Law.

It has been pointed out already that overshoot occurs in the current response due to a capacitance charging effect, the time constant being approximately $R_i C_m$. This overshoot corresponds to an exponential rise in membrane potential. Now in an excitable membrane, there exists a threshold voltage above which there is a finite probability of a transition to the excited state; thus it can happen that the onset of a transition has occurred before the clamp has had time to drive the membrane potential to a chosen level, so that the part of the current response due to excitation may be confused with

the overshoot caused by the internal resistance of the clamp system. This is evidently an undesirable state of affairs and so a criterion for a functional voltage-clamp is that its time constant be considerably less than the time required for a transition of the membrane from the resting to the excited state of the membrane. In Nitella this transition time is about 100 msecs. so that since the membrane capacitance is about 1·0 μF, a clamp internal resistance of less than 10 KΩ is required; however, since the voltage source internal resistance has to be small compared with the excitation membrane resistance and since this is about 5 KΩ anyway, then rise time difficulties are not serious in voltage-clamp experiments on Nitella.

From an experimental point of view, the analogue dealt with so far differs from an actual excitable membrane in one important aspect; the electrodes used to connect the voltage source to the membrane may easily have their electrical resistance small compared with 5 KΩ. However, in order to pass current across a biological membrane, electrodes whose resistance is comparable with the resting membrane resistance have to be used, for such electrodes are the best available. It is clear that it would be impossible to inject constant voltage pulses across the membrane using these electrodes without a loss of voltage across them. It is in order to avoid this difficulty that feedback systems must be used.

In Fig. 3e is shown the basic circuit of the voltage-clamp system used to study excitable membranes. A is a high input impedance differential amplifier and B is a source of

potential E_c whose internal resistance is less than the input impedance of the amplifier. The membrane potential is backed off by E_c and this difference is fed into the amplifier. This signal is amplified by a factor μ and inverted. The output from the amplifier is then fed back into the membrane. The system may be analysed as follows:

$$\mu(E_c - E) = E + R_s I \qquad (3.2)$$

by Ohm's Law, where R_s is the resistance of the electrodes and where E is the membrane potential. Rearranging:

$$E = E_c \frac{\mu}{1+\mu} - I \frac{R_s}{1+\mu} \qquad (3.3)$$

To a good approximation this reduces to:

$$E = E_c - I \frac{R_s}{\mu} \qquad (3.4)$$

Thus the circuit in Fig. 3e. is equivalent to that in Fig. 3f. It will be noticed that this circuit is basically the same as that in Fig. 3c. for the effect of the electrode resistances can be minimised by increasing the amplification factor of the amplifier. The actual experimental system used is shown in Fig. 3g; A and C are 'Keithley' electrometer amplifiers with differential inputs. B is a potentiometer simply producing potentials up to 200 mV on closing a switch. The membrane potential is measured with the electrometer C and displayed on an oscilloscope; the membrane current is measured and displayed on the oscilloscope using the potential across R_p.

The operation of the system was as follows. The membrane potential recorded by C was backed off to zero using B, and the output from A was fed to earth through R_w by closing the switch S. R_w was of the same order of magnitude as R, or about 1·0 MΩ. Then A was adjusted until the output current was zero. The

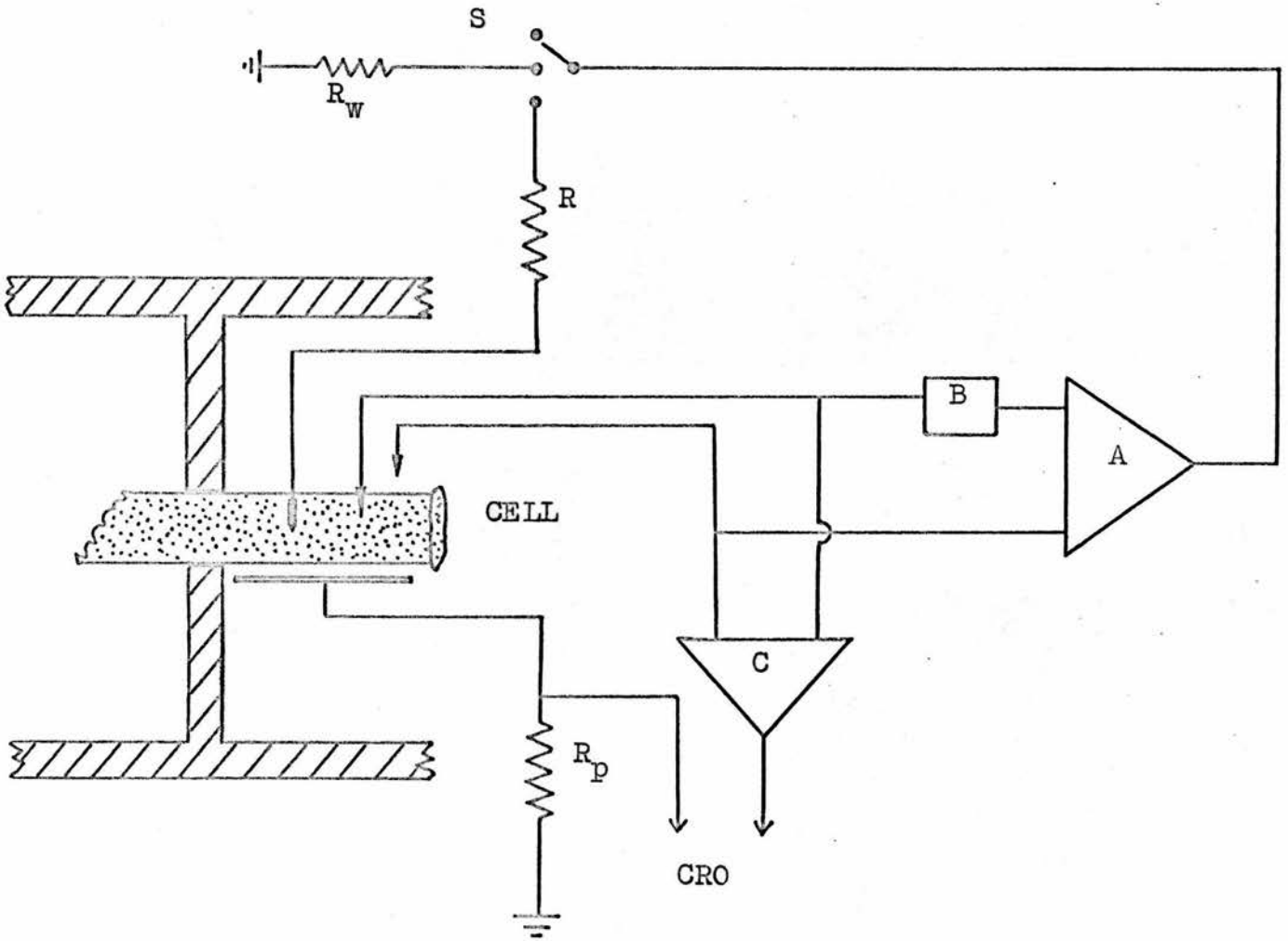


Fig. 3g The experimental electrode arrangement and voltage-clamp circuit (for description see text).

output from A was then fed into the membrane through R, thus completing the feedback loop. The gain used was 1000. Next, the resistance R was gradually decreased and this had the effect of tightening the clamp. However, if R was decreased too much the whole system oscillated. This phenomenon usually occurred when R was about 300 KΩ. Feedback systems have a tendency to oscillate but the present author has not been able to discover the exact cause of the oscillations in this particular system. Findlay (1962) has complained of the same trouble. It is desirable to be able to decrease R to about 50 KΩ, for further decrease in R can in theory have little effect upon the tightness of the clamp. In practice R was decreased until the oscilloscope current trace indicated that instabilities were about to develop; thus the clamp was pushed to its limit and operated on the fringe of instability.

During excitation small departures of the membrane potential from the clamp voltage could be observed, particularily for clamp voltages in the region of the threshold voltage. These departures were normally less than 5 mV, but for some membranes they could be as high as 10 mV. Findlay (1962) found much the same with his voltage-clamp system. The results obtained from the experiments indicate that this is partly due at least to a low dynamic resistance of the excited membrane at potentials close to threshold.

The electrodes used were a standard micro-electrode for internal voltage recording, a calomel electrode for external voltage recording, a platinum wire as external current electrode and a silver wire of 0.2 mm. diameter, tapered and tipped with AgCl, as the internal current electrode.

In order that a reasonable area of membrane may be clamped, the geometry of the cell must be considered. Nitella cells are cylindrical, about 1·0 mm. in diameter, and anything up to 10 cm. in length. Current injected at the center of a length of cell produces a displacement V_x of the resting potential at a distance x from the point of injection given by:

$$V_x = I \frac{\lambda}{2} (r_o + r_i) \frac{\cosh \frac{l - x}{\lambda}}{\sinh \frac{l}{\lambda}} \qquad (3.5)$$

This equation is well known and is most familiar as the relation governing the time independant behaviour of a leaky transmission line. It has already been used in Chapter 11. λ is the space constant and is a measure of the spread of current along the membrane; the greater λ is, the greater is the spread. λ is given by: $\lambda^2 = \frac{r_m}{r_o + r_i}$ where r_m is the resistance of the membrane per unit of length. The length of the cell is $2l$ and I is the current injected.

For a long section of cell, equation (3.5) may be approximated by:

$$V_x = I \frac{\lambda}{2} (r_o + r_i) e^{-\frac{x}{\lambda}} \qquad (3.6)$$

and if V_o is the displacement of the resting potential when x is zero then: $V_\lambda = V_o \, e^{-\frac{x}{\lambda}}$ (3.7)
Thus at a distance given by: $x = \lambda$, V_x has dropped to almost a quarter of the value of V_o. Clearly a region of uniform potential will only be found up to distances considerably less than the space constant from the point of injection. The space constant of Nitella translucens has been measured by Williams, Johnston and Dainty (1964), by Hogg

(private communication) and by the present author (Chapter 11); it is around 3·0 cm. However in excitable membranes the membrane resistance can drop to about one tenth of its resting value and if this happens in Nitella then since λ depends on the square root of the membrane resistance it will drop to about 1·0 cm. Hence voltage-clamp experiments are not meaningful when performed on a cell of length exceeding about 2·0 cm. This has been emphasised by Cole (1949) and Kishimoto (1964). In the present experiments, this restriction was scrupulously observed, either by using short cells, or end lengths of long cells externally insulated from the rest of the cell by means of a two compartment bath. A schematic layout of the electrode system and clamped cell section is shown in Fig. 3g. The electrodes are arranged in much the same way as in Kishimoto's system (1964). A critisism of this layout would be that it does not allow a distinction to be drawn between the electrical properties of the composite membrane, consisting of the cell wall, the cytoplasmic membrane and the vacuolar membrane, and the cytoplasmic membrane alone. According to Findlay and Hope (1964a) both membranes in Chara are excitable, and these authors (1964a) claim to have been able to apply a voltage-clamp to the cytoplasmic membrane alone. However they managed to insert into the cell an axial current electrode thus eliminating the very difficult problems involving cable theory. In all of the experiments described here, the cytoplasmic membrane, vacuolar and cell wall are taken as being lumped together in series, clamped and referred to as the 'membrane'.

The External Solutions

The ionic solutions used in the experiments were made up with the aim of determining the effect of Na, K, Ca and Cl on the current response of the membrane. The external ionic solution was normally a standard solution referred to as Ca-APW. This differs from the standard solution used in the previous chapter in that the CaCl concentration is 1 0 mM instead of 0 1 mM. This Ca APW was used as a standard solution because the author had observed during the course of the experiments described in the previous chapter that cells in solutions deficient in Ca were rarely excitable. During the experiments the external solution could be changed to any of what will be referred to as: High Cl, Low Ca, Medium Na and K and High K and Low Na. The ionic compositions of these solutions are given in Table 3a.

IONIC COMPOSITIONS OF SOLUTIONS USED IN THE EXPERIMENTS

	mM KCl	mM NaCl	mM CaCl$_2$
Ca-APW	0·1	1·0	1·0
High Cl	0·1	10·0	1·0
Low Ca	0·1	1·0	0·1
Medium Na and K	0·5	0·6	1·0
High K and Low Na	1·0	0·1	1·0

Table 3a.

The Experiments

Two sets of experiments were performed. In the first set, membrane data was obtained for cells in APW, High Cl and Low Ca external solutions; in the second set exactly the same experiment was performed with cells in Ca-APW, Medium Na and K and High K external solutions. Altogether, the membranes of 45

cells were successfully clamped; experiments on another 80 cells were not completed for technical reasons so that the casualty rate in this work ran at about 70%.

Injection of pulses was always commenced about one hour after a solution change to allow for the re-establishment of a steady state, and since, due to the relatively long refractory period of the membrane, pulses could be usefully applied not more often than every ten minutes, the time required to complete an experiment after it had been set up was about six hours. For this reason the experiments with Na and K were performed separately from those with Ca and Cl.

3.23 Results

Ca-APW as External Solution

Typical current responses of a membrane to a series of polarising clamp voltages are shown in Fig. 3h. In this particular example the membrane potential was 60 mV and the action potential was 80 mV in size. The membrane capacitance charging and discharging current transients may be seen at the beginning and end of each current response. Incidently these transients confirm that the membrane capacitance of the membrane of Nitella translucens is of the order of 1·0 μF cm^{-2}. in agreement with the results of Williams, Johnston and Dainty (1964). The rise time of the voltage pulse is a few msecs.

The first depolarisation of the membrane is not sufficiently large to cause excitation and the current response is outward and constant. However, a polarisation of 25 mV produces a small transient outward current followed by a large transient inward current, which is in turn followed by a steady outward current.

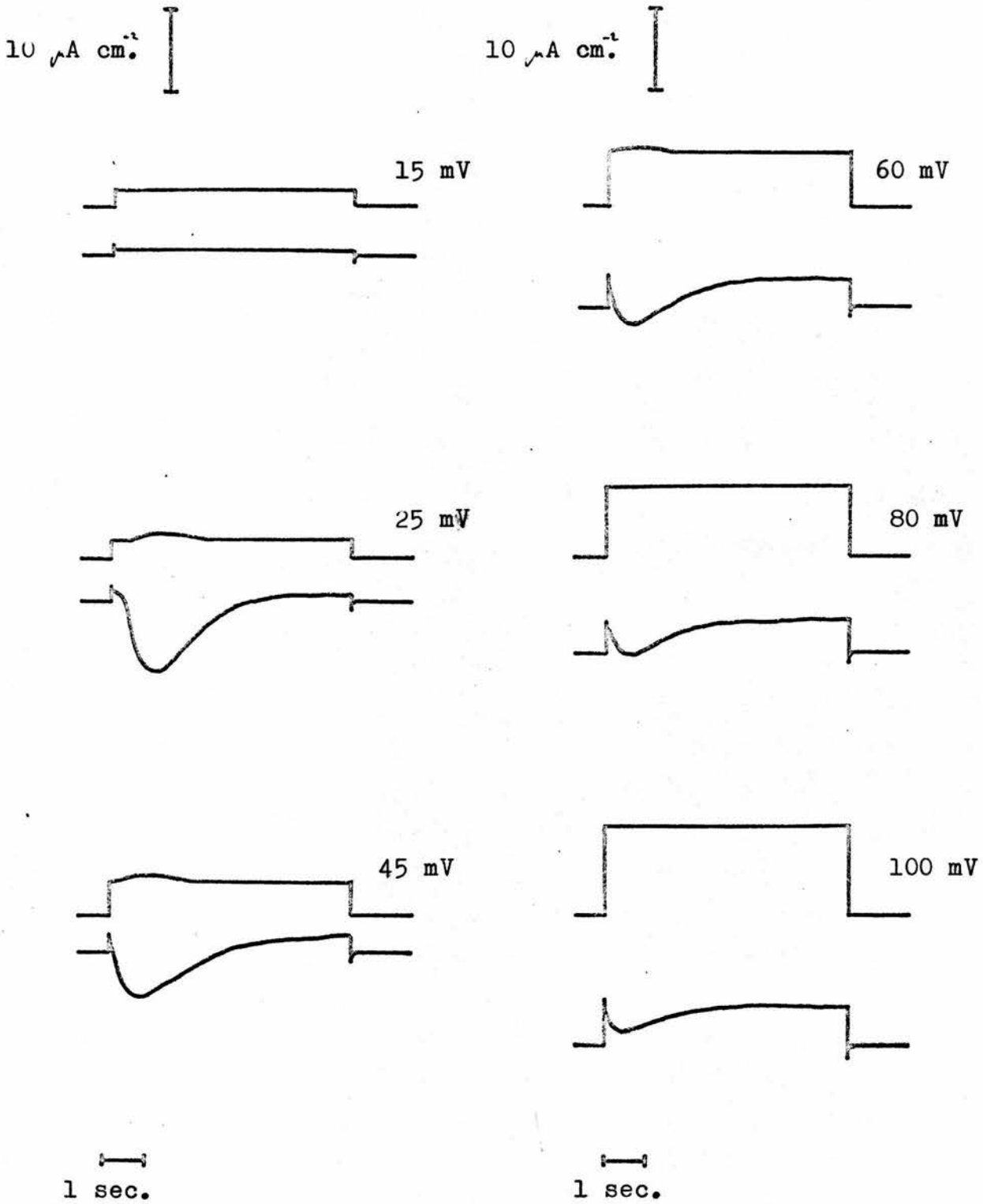


Fig. 3h Membrane current responses to depolarising voltage pulses.

With stepwise increase in membrane depolarisation both the initial outward transient current and the final outward steady current increase in a stepwise manner at all times. On the other hand, the transient inward current decreases with increasing depolarisation to become zero when the depolarisation is 80 mV. The value of the depolarisation is at this stage numerically equal to the value of the action potential. With further depolarisation this transient inward current becomes a transient outward current. When the membrane is hyperpolarised, the current is inward and constant and it increases with increasing membrane hyperpolarisation. By and large the responses are similar to those of other excitable membrane. It must of course be remembered that the time scale is in seconds.

Unfortunately, the membrane was imperfectly clamped for small depolarisations, as indicated by the slight unevenness in the voltage trace. This was probably due to the inability of the clamping system to cope with the low resistance of the membrane in the excited state at these potentials.However it is unlikely that this will affect the overall reliability of the results; possibly with a perfect clamp the transient inward current would be a little larger at potentials near to the threshold potential. For greater depolarisations the membrane potential was constant during excitation.

It is convenient to regard the membrane as possessing two states: the resting state and the excited state. The excited state is short-lived, having a life-time of about 0·5 seconds; this is about 1000 times longer than the life-time of the excited state of nerve membranes. The transitions of the

membrane from the resting state to the excited state and then back again to the resting state are illustrated in Fig. 3i. It is clear that the variation of the peak transient current with the clamp voltage gives the current voltage characteristic of the membrane in its excited state. The current voltage characteristic of the membrane in its resting state is obtained from the variation of the steady outward current with polarising clamp voltage. It is difficult to interpret the currents flowing during the periods of transition of the membrane from the resting to the excited state and from the excited to the resting state. The relatively long transition times seem to indicate that the membrane passes through a series of intermediate states during the transitional periods. Each of these intermediate states might be described by a current voltage characteristic. In view of the difficulties involved no effort will be made to study these intermediate states, but an attempt to do so is described in a later section.

The current voltage characteristics of the membrane for both the resting and the excited states are shown in Fig. 3j. The data was obtained from the current responses for different membrane potentials shown in Fig. 3h. The lower curve describes the membrane in its excited state. From this graph it can easily be seen that voltage-clamping merely involves the production of 'horizontal' transitions, or transitions at constant voltage, between the resting and excited state of the membrane. Also the action potential is seen to be just a transition between the resting and excited state at zero current.

In the resting state, the current voltage curve is

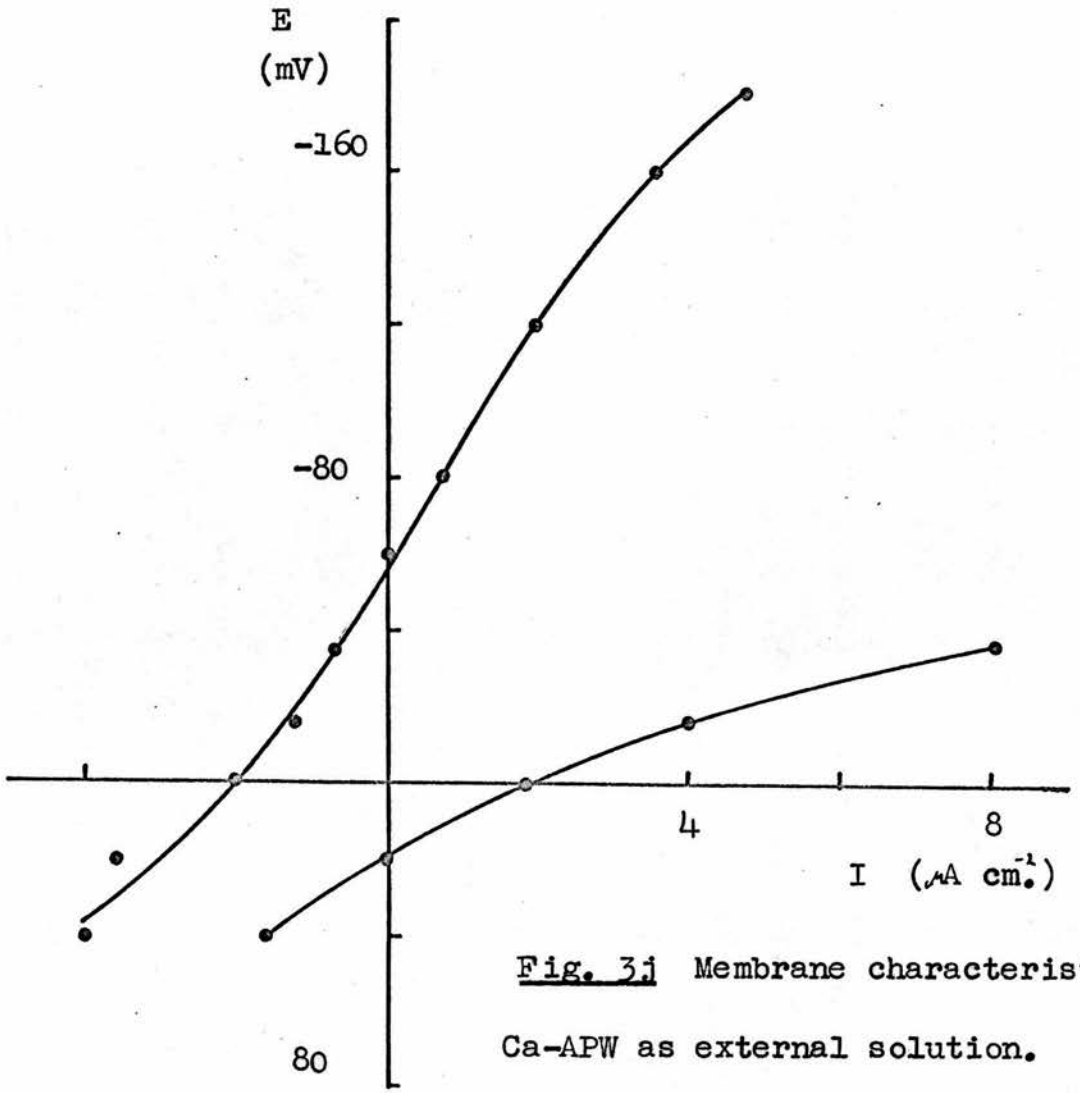


Fig. 3j Membrane characteristics with Ca-APW as external solution.

approximately a straight line but often at high levels of polarisation and hyperpolarisation the dynamic membrane resistance tends to decrease so that the membrane exhibits rectification phenomena. The resting resistance, R_m, is taken as the slope of the curve at I=0. In this example R_m is found to be 28 $K\Omega cm^2$. The membrane in the excited state behaves in a somewhat similar fashion. The dynamic resistance is greatest for small membrane currents and least for high membrane currents. The excitation resistance is taken as the slope of the curve at I=0. The value here is 13 $K\Omega cm^2$. However, the lowest resistance of this membrane occurs at about threshold potential and is found to be 4ΩK cm^2. Experimental errors in resistance measurements are not greater than 10%.

These characteristics appear to have a lot in common with those of vacuum tube and transistor devices. The state of such a device, as described by its current voltage characteristics, can be varied by changing the grid voltage in the case of a triode, or the base current in the case of a transistor. The state of an excitable membrane cannot be varied at will, but the membrane does undoubtedly possess at least two states. There are in existance many different kinds of excitable membranes and detailed information is available only for a few. Similarily there are many different kinds of vacuum tube and transistor devices in existence. It is common practice to describe the electrical behaviour of these devices in terms of idealised current voltage characteristics, idealised electrical parameters and equivalent circuits; there is clearly a close correspondence between the two methods. In the absence of any

other proposals, it is intended to follow this practice here in dealing with excitable membranes; in the author's experience it introduces a simplicity which is useful and reliable in both teaching and research.

The simplest set of characteristics is shown in Fig. 3k. It simply consists of two straight lines of different slope; the upper curve corresponds to the resting state and the other to the excited state. The points at which the curves cut the voltage axis gives the membrane potential in the resting and excited states. The principal parameters used to describe the electrical properties of the membrane are given in Table 3b. An equivalent circuit representation is shown in Fig. 3l.

EXCITABLE MEMBRANE PARAMETERS

PARAMETER	SYMBOL
Resting resistance	R_m
Excitation resistance	R_e
Resting potential	E_m
Excitation potential	E_e
Membrane capacitance	C_m

Table 3b.

It will be seen that a simpler correspondence with the parameters is achieved if a series circuit is used instead of a parallel circuit. When the switch is closed the circuit represents the membrane in its excited state.

The results of experiments on 45 cells are shown in Table 3c. C_m was not measured in these experiments. The mean value of 24 $K\Omega cm^2$. for R_m is in close agreement with the value obtained by the short-circuit method described in the previous

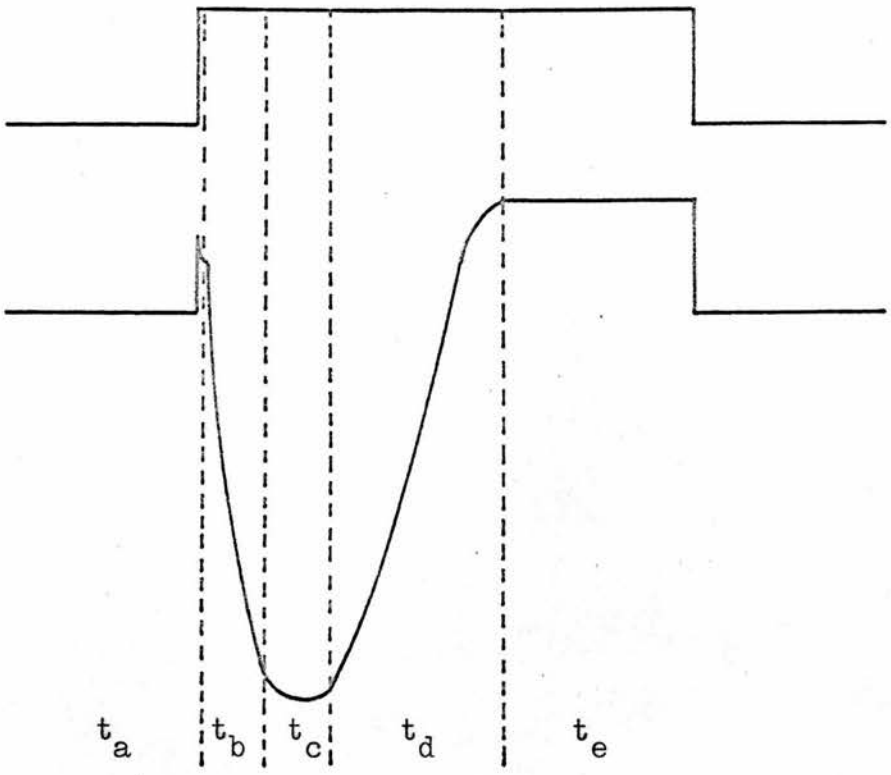


Fig. 3i Time analysis of membrane activity: during t_a and t_e the membrane is in the resting state, during t_c it is in the excited state and t_b and t_d are transitional periods.

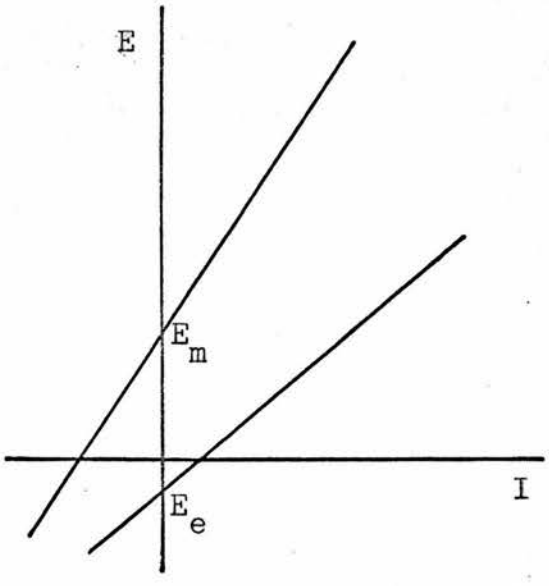


Fig. 3k Idealised membrane characteristics.

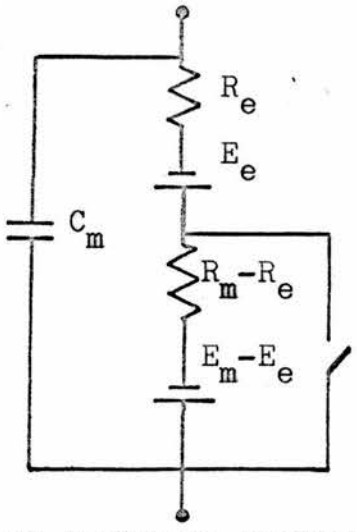


Fig. 3l Membrane analogue.

Table 3c. MEMBRANE PARAMETERS IN ᵃAPW

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm².)	R_e (KΩcm².)
1	-63	- 2	20	5·0
2	-73	10	17	6·7
3	-75	8	13	4·3
4	-52	22	16	8·3
5	-64	17	13	8·0
6	-69	24	20	8·5
7	-80	3	23	6·6
8	-64	16	15	2·0
9	-62	10	13	2·9
10	-40	28	9·0	0·9
11	-60	23	10	2·5
12	-52	0	6·6	2·2
13	-53	5	6.7	1.7
14	-68	- 4	8.3	1·0
15	-63	20	6·7	2·5
16	-86	-20	40	6·6
17	-77	12	30	3·3
18	-42	- 7	5·0	3·3
19	-41	20	13	6·6
20	-45	25	50	5·0
21	-78	15	62	5·0
22	-63	20	90	6·6
23	-60	20	28	13·0
24	-35	20	25	12·0
25	-60	5	15	3·3

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm².)	R_e (KΩcm².)
26	-48	10	30	4·3
27	-98	-40	80	10·0
28	-60	12	40	4·0
29	-60	5	50	6·6
30	-64	4	40	5·0
31	-74	-10	33	4.0
32	-60	6	40	3·3
33	-74	10	20	4·0
34	-60	2	65	8·0
35	-68	- 1	11	3·3
36	-70	20	40	3·4
37	-64	10	60	5·7
38	-44	-16	4·0	3·3
39	-64	10	20	1.6
40	-88	-14	15	3·3
41	-58	5	13	5.0
42	-50	25	10	2·5
43	-40	2	12	6. 0
44	-61	-20	20	3·3
45	-85	-10	40	10·0
Mean	-63	5	27	5·0

Table 3d. MEMBRANE PARAMETERS IN LOW Ca.

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm²)	R_e (KΩcm²)
1	-83	-27	15	3·3
2	-97	-12	15	5·0
3	-88	-19	13	3·7
4	-69	0	13	8·3
5	-77	-27	10	6·0
6	-110	15	6·0	2·5
7	-85	3	26	10·0
8	-64	- 2	8·6	2·0
9	-85	- 5	23	5·0
10	-55	7	8·5	2·8
11	-86	23	10	3·3
12	-78	-26	6·7	2·0
13	-80	-20	8·0	2·0
14	-90	27	10	1·0
15	-106	-20	8·0	1·4
16	-80	-20	20	6·7
17	-140	-73	60	15·0
18	-75	- 6	25	6·0
19	-95	-30	50	6·6
20	-106	-52	11	6·0
21	-1o7	-35	43	5·0
22	-45	- 2	34	14·0
23	-110	-50	51	8·0
24	-60	0	64	12·0
25	-61	-20	50	14·0
Mean	-85	-15	24	6·1

Table 3e. MEMBRANE PARAMETERS IN HIGH Cl.

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm².)	R_e (KΩcm².)
1	-63	-17	15	4·0
2	-75	7	20	3·6
3	-77	7	14	5·0
4	-45	1	7·5	3·0
5	-71	9	10	7·0
6	-96	25	5·0	2·5
7	-80	2	25	3·3
8	-70	11	7·5	2·5
9	-60	16	20	5·0
10	-47	15	9·0	0·9
11	-65	15	6·2	1·9
12	-68	-12	6·0	1·7
13	-60	12	10	2·1
14	-70	2	10	0·8
15	-77	6	10	1·7
16	-56	3	30	4·3
17	-98	-40	80	10·0
18	-60	12	23	8·3
19	-78	- 6	50	7·5
20	-76	- 3	21	7·0
21	-70	- 9	44	3·3
22	-40	6	16	6·0
23	-80	10	50	10·0
24	-54	14	49	5·0
25	-64	4	24	4·0
Mean	-66	4	23	4·4

Table 3f. MEMBRANE PARAMETERS IN MEDIUM Na AND K.

Exp.	E_m (mV)	E_e (mV)	R_m (Kλcm.)	R_e (Kλcm.)
26	-90	20	26	15·0
27	-63	0	6·0	3·3
28	-35	3	6·6	3·3
29	-60	17	8·0	5·0
30	-52	30	50	3·3
31	-90	6	30	6·0
32	-80	32	40	4·2
33	-73	10	60	13·0
34	-60	20	30	4·4
35	-64	17	22	5·3
36	-82	- 3	50	3·3
37	-60	20	40	6·6
38	-54	10	12	2·5
39	-67	- 8	20	5·0
40	-84	-12	12	5·o
41	-73	- 2	15	3·1
42	-56	23	20	2·5
43	-40	0	20	6·6
44	-62	-21	40	3·3
45	-90	-10	40	13·0
Mean	-67	8	27	5·6

Table 3g. MEMBRANE PARAMETERS IN HIGH K.

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm^2)	R_e (KΩcm^2)
26	-82	20	30	10·0
27	-65	12	13	5·0
28	-29	17	6·6	3·3
29	-63	12	25	8·0
30	-60	65	50	7·5
31	-105	23	35	7·0
32	-85	29	60	12·0
33	-78	11	40	15·0
34	-30	12	20	8·0
35	-61	-19	16	3.7
36	-95	-10	33	5.0
37	-60	12	13	7·5
38	-50	12	10	2·5
39	-64	40	15	2·0
40	-60	- 8	12	3·0
41	-85	9	20	2·5
42	-58	-10	5·0	3·4
43	-40	- 6	6·7	6·7
44	-62	-21	40	3·3
45	-90	0	40	15·0
Mean	-66	11	25	6·5

chapter. The low value of the resting potential was due to the high concentration of Ca in the external solution.

Low Ca and High Cl external solutions.

Current voltage characteristics of a typical cell in APW, in Low Ca and in Cl solution are shown in Fig. 3m. There seems to be very little difference between the three sets of characteristics, except that in Low Ca solution the characteristics are shifted 'upward' along the voltage axis making E_m and E_e about 20 mV more negative. The membrane parameters for both Low Ca and High Cl solutions resulting from measurements on 25 cells are listed in Tables 3d and 3e. On average the membrane resistances are unaffected by these solutions. Again the values of E_m and E_e are hardly affected by the High Cl solution, while E_m and E_e are each 18 mV more negative in Low Ca solution. These results are significant because if Cl is involved in the action potential then High Cl solution should affect E_e. On the other hand, the fact that the Low Ca solution affects both E_m and E_e by exactly the same amount lends support to the hypothesis advanced in the previous chapter that the cell wall exhibits a masking effect in any experiments involving changing the external solution.

Medium Na and K and High K as the external solutions.

From Fig. 3n. it may be seen that the characteristics of a typical cell are much the same in Medium Na and K and in High K as in APW. It is clear that these solutions have little effect on the characteristics. Membrane parameters derived from experiments on 20 cells are shown in Tables 3f and 3g. The parameters are not significantly affected by the solutions,

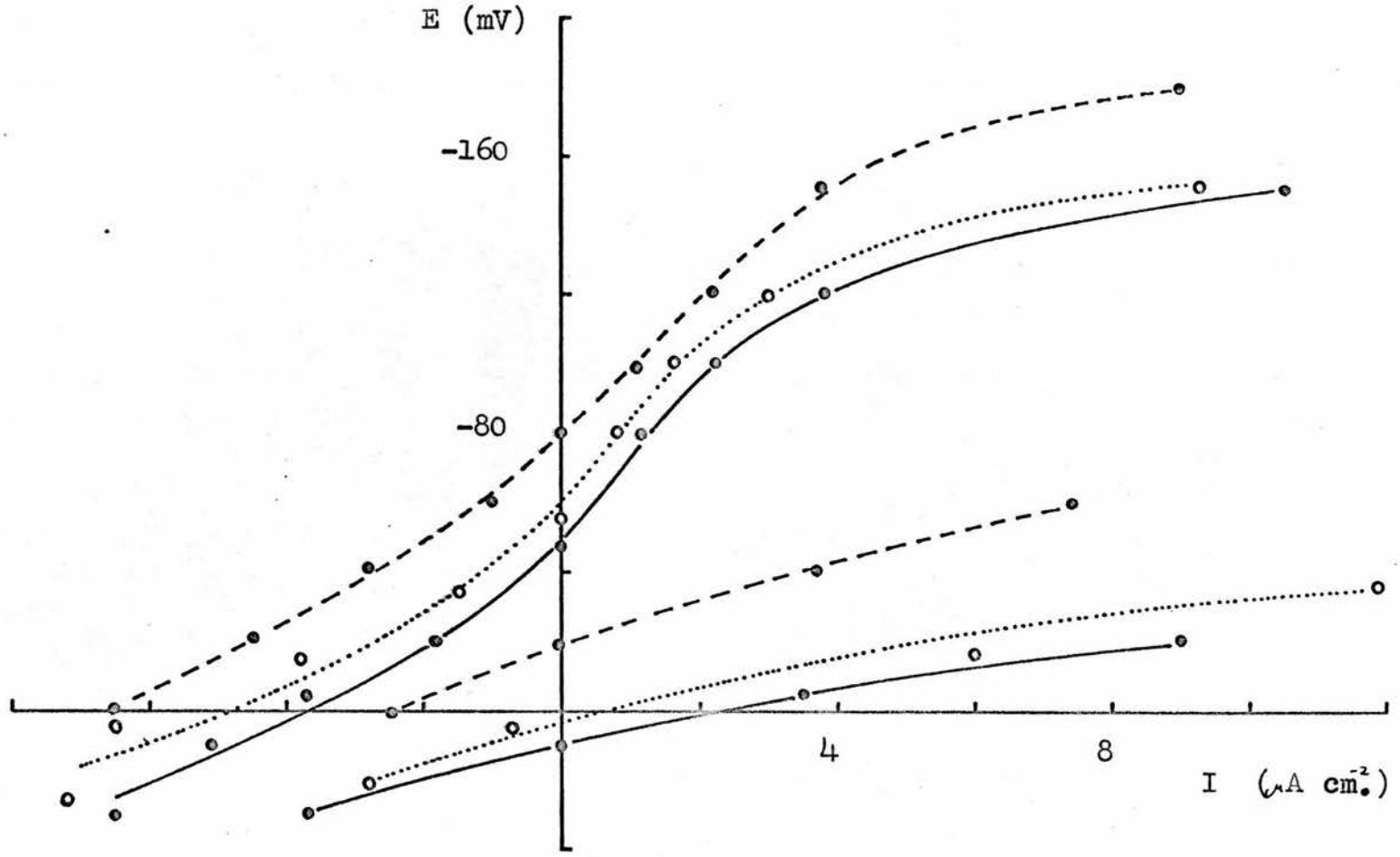


Fig. 3m Membrane characteristics in Ca-APW (full curve), in High Cl (dotted curve) and in Low Ca (dashed curve).

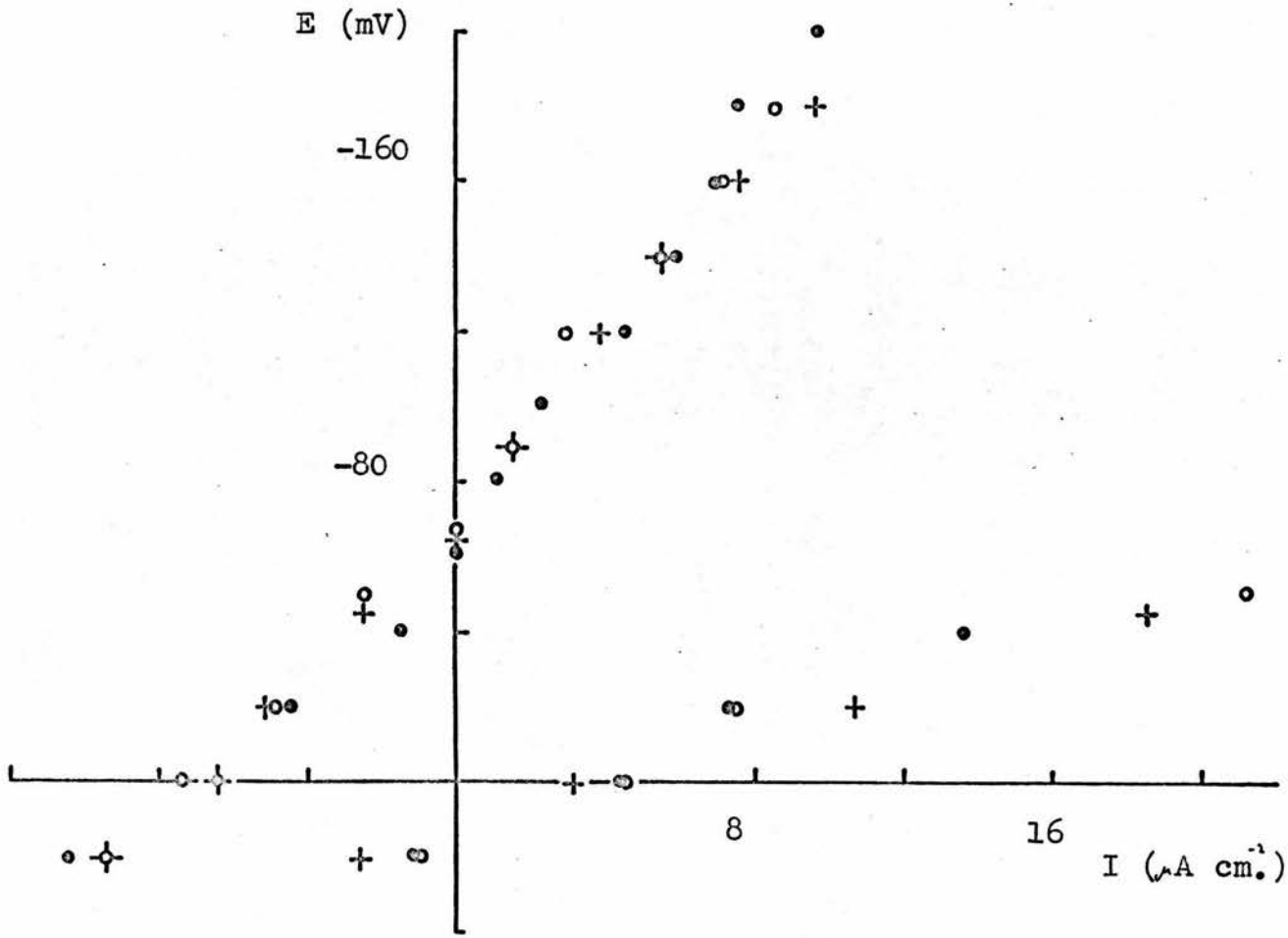


Fig. 3n The relation between membrane potential and membrane current in Ca-APW (closed circles), in Medium Na and K (open circles) and in High K (crosses).

which is in marked contrast to the behaviour of nerve membranes.

3.24 Discussion

The membrane resistances values are in good agreement with those of other workers. The value of 24 KΩcm^2 . for R_m compares favourably with the values obtained by Findlay (1959), by Williams, Johnston and Dainty (1964) and by Kishimoto (1964). The value of 5·0 KΩcm^2. for R_e is in agreement with that of Findlay (1962), Kishimoto (1964) and Cole and Curtis (1938).

One of the immediate problems in this kind of work is the identification of the clamp currents in terms of the charge carriers which are transported across the membrane in the resting state, in the excited state and during the transitional periods. In a given situation it is often difficult to determine just what charge carriers are responsible for a given current. In the present situation, electrons and holes are possibilities which must always be borne in mind but it is known from ion flux experiments that some current across biological membranes is certainly carried by ions. Thus the problem reduces to determining which ions carry the various currents observed in the course of voltage-clamp experiments. The most satisfactory method of doing this involves the determination of the ionic fluxes of Na, Ca and Cl across the membrane when the clamp currents are flowing. A simpler method involves measuring the effect of changes in the concentration of these ions on E_m and E_e. The extent to which a current is distributed among these ions is deducible from the ionic transport numbers which may be obtained from the transport number equation used in the previous chapter.

The present work has shown that a ten-fold decrease in Ca concentration produces on average a 18 mV decrease in E_m and E_e. In Nitella Findlay (1962) has found that Ca produces a 30 mV change in E_e per ten-fold change in external concentration but has no effect on E_m. In contrast, Kishimoto (1964) finds that Ca has no effect on either E_m or E_e in Nitella. Adelman and Moore (1961) showed that Ca had little or no effect on E_m or E_e in squid giant axon. Julian, Moore and Goldman (1962) have found that with lobster giant axon E_m increases and E_e decreases in Low Ca solution. In Nitella translucens the present work seems to indicate that the effect of Ca is due to the cell wall for it is hard to see how the fact that both the resting and the excited state of the membrane are affected in exactly the same manner can be explained in any other manner. The clamped membrane may be regarded as being composed of an excitable membrane in series with a membrane of low electrical resistance but selectively permeable to Ca, so that changes in E_m due to excitation are located across the plasmalemma while changes in E_m due to Ca concentration changes are located across the cell wall.

These experiments show that Cl has no effect on either E_m or E_e. Findlay and Hope (1964b) find that Cl has a small effect on E_e in Chara; these authors have also reported (1964) that the Cl efflux increases during the action potential. (Gaffey and Mullins (1958) have found that in Chara globularis the efflux of Cl increases during the action potential and Mullins (1963) has found the same effect in Nitella). Kishimoto (1964) finds a considerable reduction in the size

of the action potential in Nitella following a ten-fold increase in Cl concentration. And in general it has been found that Cl has no effect on E_m or E_e in nerve membrane. Thus the evidence strongly suggests that during the action potential in Nitella and Chara the membrane Cl conductance increases. The lack of any effect of Cl on E_e in the present experiments is probably due to the cell wall which must be a poor conductor of Cl compared with Ca. However, the conductivity of the cell wall is large compared with that of the plasmalemma so that the cell wall will not affect the Cl efflux experiments to any great extent. The short-circuit experiment described in Chapter 11 provides additional evidence in support of the Cl hypothesis.

The effect of Na and K on E_m and E_e is negligible according to the present work. There are no reports in the literature of these ions having any effect on the action potential in Nitella or Chara. In sharp contrast, it is well established that nerve membranes increase their Na and K conductances in the excited state. In fact it might be remarked that it seems strange that the excitable membranes of animal cells should be so fundamentally different from those of plant cells.

In conclusion, it seems probable that the membrane of Nitella becomes a Cl conductor during excitation and that the cell wall is probably the greatest single stumbling block to further progress. Otherwise the electrical properties of Nitella membranes appear to be similar to those of other excitable membranes.

Lastly, the similarity of an excited membrane to a multi-state electronic device has been emphasised and employed as a

guide in the design of the experiments and in the presentation of the results. This simplification is used in the design of the other experiments described in this chapter.

3.3 CONSTANT CURRENT EXPERIMENTS

3.31 Introduction

The Two State Model

In the previous section, the behaviour of the membrane of Nitella translucens under a voltage-clamp was studied. It was found to behave electrically like other excitable membranes; however, it was also pointed out that the phenomenon of excitability could be more simply described if an excitable membrane were regarded as possessing two states, the resting state and the excited state, so that excitability was a consequence of the membrane undergoing transitions between these states. Thus clamping the membrane potential causes all transitions to occur at constant voltage.

However, according to the model, the membrane could also be forced to undergo transitions at constant current. This may be clearly seen from the analogue in Fig. 31. In fact, as was remarked in the last section, the action potential as commonly observed is nothing more than a membrane transition at constant but zero current. Thus from a study of transitions at constant current it should be possible to obtain the current voltage curves for the two states of the membrane, and the curves should then be the same as those obtained from the voltage-clamp experiments. There is one major drawback in this method of obtaining the membrane characteristics at constant current. Clearly the membrane will only become excited when the membrane potential is above the threshold potential, or, in other words, when the membrane current is outward; thus it is not possible to obtain the portion of the current voltage

characteristic which describes the excited state of the membrane when the current is inward. In this respect, the voltage-clamp technique is superior to the current-clamp technique.

Studies of the two states of the membrane with the current-clamp technique are described in this section and the membrane current voltage characteristics so obtained are in good agreement with those obtained from the voltage-clamp experiments described in the previous section. The author is not aware of any previous systematic attempt to study both the resting and excited state at constant current. However there may be very good reason for this. In order that these experiments be meaningful, it is necessary that the time constant of the membrane be less than the time required for the membrane to switch from the resting to the excited state. This condition is fullfilled only in the excitable membranes of the giant algal cells of the Characeae.

The Membrane at High Levels of Hyperpolarisation

It has been shown by Coster (1965) that when the membranes of both Chara australis and Nitella are hyperpolarised to very high levels, the dynamic membrane resistance abruptly drops to a very low value, so that the membrane potential becomes almost independent of the membrane current. Such behaviour is similar to that of a Zener diode and as already mentioned Coster has shown theoretically that a membrane composed of an anion and cation exchange membrane in series would be expected to have properties similar to those of a p-n junction. However, Coster's theoretical analysis shows that 'punch-through' (which occurs in transistors when the base region is depleted of charge carriers)

and not Zener breakdown is responsible for breakdown in a thin c-a membrane. This would then explain the observed effect in Chara and Nitella.

Experiments to confirm Coster's work are described in this section. The present author has failed to observe the abrupt breakdown effect described by Coster, but has found a gradual decrease in dynamic membrane resistance.

3.32 Methods

The Electrical System

Square pulses of current of variable magnitude, duration and polarity could be made to flow across the membrane as desired. The current was injected by means of a pair of Ag/AgCl electrodes. Passing the current through a resistance of 10 MΩ ensured that the pulses were square, since the combined resistance of the current electrode and the membrane was about 100 KΩ. All of the pulses were manually switched.

The membrane current was measured as the potential across a 10 KΩ resistor in series with the current electrodes; this potential was then displayed on an oscilloscope. The membrane potential was recorded by a micro-electrode inside the cell and a salt bridge electrode outside the cell, and then displayed on the oscilloscope.

The arrangement of the electrodes and the kind of electrodes used in the experiments were exactly the same as for the voltage-clamp experiments. The same consideration about cable theory apply and so by using the same two compartment bath as in the voltage-clamp experiments it was possible to confine the membrane current to a length of cell never exceeding 2·0 cms.

The Experiments

Three series of experiments were performed. The first two were performed with the aim of confirming the results of the voltage-clamp experiments; the last series was performed in order to check the work of Coster (1965).

In the first set current clamps were applied to 10 cells bathed alternatively in Ca APW, Low Ca and High Cl solution, and in the second set the first set was repeated with Ca APW, Medium Na and K and High K as alternate bathing media. These solutions were the same as those used in the voltage-clamp experiments. During the course of these two sets of experiments, polarising current pulses were applied to the membrane every 10 minutes in order to allow the membrane to regain full excitability following an action potential; hyperpolarising pulses were applied about every two minutes. However as will be mentioned again later, the act of switching a hyperpolarising current off was found to be very often capable of causing excitation and if this happened then 10 minutes were allowed to elapse before the next hyperpolarising pulse was injected.

In the last set of experiments the cell were bathed in Ca APW and Ca free APW as alternate bathing media; both of these solutions were used in the experiments described in Chapter 11. Hyperpolarising pulses were applied every few minutes and at high levels of hyperpolarisation the duration of the pulses had to be kept short, not longer than about 20 seconds, for otherwise the cell died. In these experiments the current and potential were not displayed on the osilloscope but simply

read from the dials on the electrometer amplifiers. This was done because transient potentials were not required and also because of the greater accuracy involved in taking the readings from the electrometer dials when such large voltage ranges were involved. The cell membranes were hyperpolarised down to 300 mV below the resting potential. It is clearly important to know whether the cells were still alive following the experiments; thus cells were always tested following experiments and were taken as being alive if they were still excitable.

3.33 Results

Experiments in Ca APW

A typical series of potential responses to a series of stepwise increasing current is shown in Fig. 3o. A current pulse of small magnitude produces a potential response which is similar to that of a resistance capacitance network. Increasing the magnitude of the current pulse causes the membrane to become excited during the period of the pulse. Thus the voltage response to these current pulses is non-linear. For the smaller membrane currents the potential response profile is very similar to the action potential profile, as it is commonly observed; a large initial transient potential is followed by a small steady potential. With increasing magnitude of current pulse the peak transient potential and the final steady potential both increase; the final steady potential increases more quickly.

A series of potential responses to a series of hyperpolarising pulses are shown in Fig. 3p; this series and the series shown in Fig. 3o. were obtained from the same cell. With the small current pulse the potential response was steady

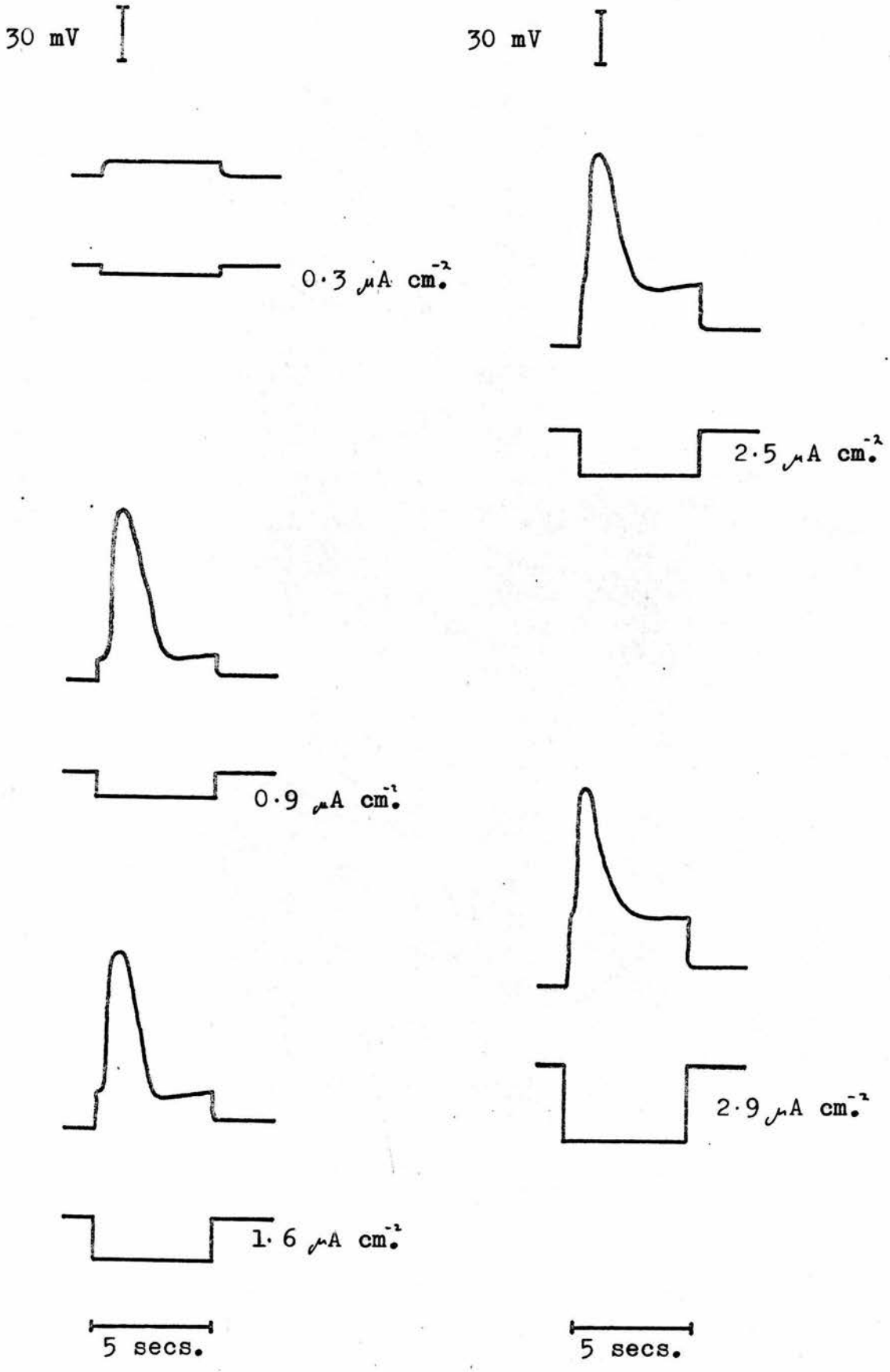


Fig. 3a Membrane potential responses to depolarising current pulses.

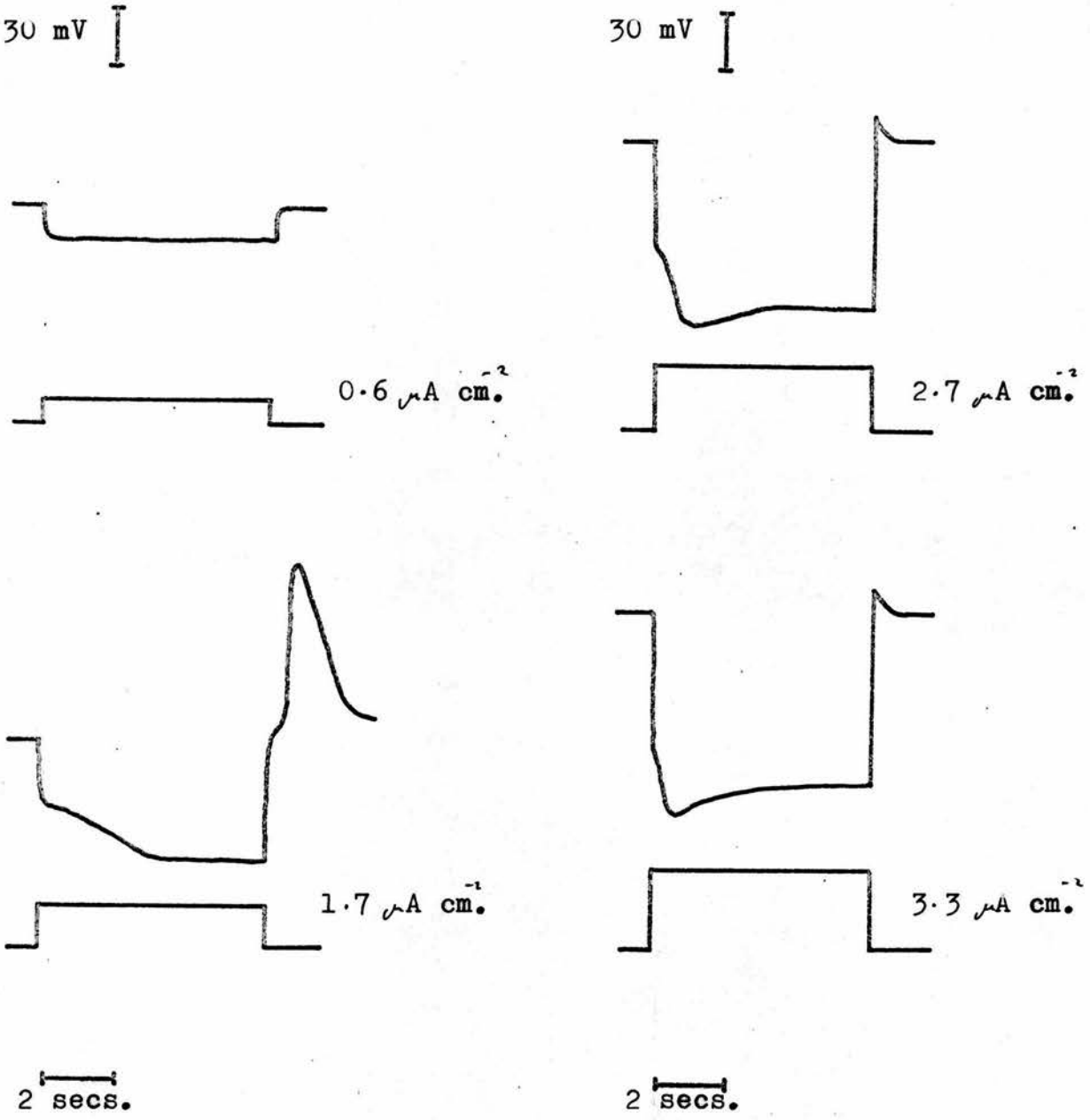


Fig. 3p Membrane potential responses to hyperpolarising current pulses.

except for the initial and final capacitance charging and discharging transients. This was typical of all the cells studied. For larger pulses of current, there is an initial potential transient and this is followed by a final steady potential. This potential transient could last up to about 6 seconds, and the average transient potential was significantly less negative than the final steady potential. However, this type of transient did not always occur and was not always reproducible using the same experimental material. The effect could be observed in about one third of the cells studied. The effect was further investigated and it was found that this type of transient usually occurred during the refractory period of the membrane; in other words, this unusual response could most frequently be obtained following an action potential. This is hardly unexpected, since the membrane resistance must be steadily increasing from R_e to R_m during the refractory period. However, this does not explain why the effect sometimes occurred when excitation was known not to have occurred for at least 10 minutes. Possibly the cells involved had particularily long refractory periods. With increasing magnitude of current pulse, the initial transient already described gave way to another initial transient of similar duration; during this transient the average potential was more negative than when the potential was steady. This transient always occurred, and usually appeared when the steady potential was more than 40 mV more negative than the resting potential. These transients could be very large; for example a membrane which responds to a square current pulse with a steady

potential of 80 mV more negative than the resting potential could have a peak transient potential 120 mV more negative than the resting potential. It is not possible to attribute this transient to any defect in the current pulse, for it can be observed that any variation in the current during the potential is less than 5%. Another transient potential could be observed, whenever the current was switched off. If the membrane is hyperpolarised to a potential more negative than the resting potential by about 40 mV or more, then on switching off the current the potential returned, not to the resting value, but to a value as large as 20 mV more positive. This depolarisation was very often capable of producing an action potential, thus indicating that the potential transient caused by switching off the current occurred across the plasma membrane, and not, as is a possibility, across the cell wall or in the cytoplasm. Thus it seems probable that the potential transient which occurs when the current is switched on is also located across the plasma membrane. An example of an action potential produced by a hyperpolarising pulse may be seen in Fig. 3p. This phenomenon is known to occur in nerve (Ooyama and Wright, 1961) and is called 'anode-break' excitation. The phenomenon has been explained on the basis of the Hodgkin-Huxley hypothesis; it is thought that when the membrane is hyperpolarised, the K permeability decreases so that if the membrane undergoes a rapid depolarisation, the potential will tend to rest at a level more positive than the resting potential, and this could bring about the regenerative series of changes leading to excitation.

The current voltage characteristics of the membrane in the resting state were obtained from the variation of the steady membrane potential with the applied current. The excited state characteristic was obtained from the variation of the peak transient potential with polarising current. It is clear that unless the time of transition of the membrane from the resting to the excited state is greater than the membrane time constant, the potential of the membrane in the excited state will be confused with the initial potential transient due to the charging of the membrane capacitance. In Nitella translucens the transition time is rarely less than 100 msec., whereas the time constant is usually less than this value. However, a few cells do exhibit transition times about equal to the membrane time constant so that some error may be involved with a few of the cells. It is for this reason that the experiment would not be possible with nerve cells.

Typical current voltage characteristics obtained from the oscilloscope traces are shown in Fig. 3q. The curve for the resting state is similar to a typical curve deduced from voltage-clamp experiments. The curve for the membrane in the excited state can unfortunately, only be deduced for outward membrane current ranges owing to the fact that the membrane cannot be excited by an inward current. In this particular example the values of R_m and R_e were 33 and 6·7 $K\Omega cm^2$. respectively and the values of E_m and E_e were -70 and +10 mV respectively. In order to deduce R_e and E_e the curve for the excited state was produced to cut the voltage axis. In Table 3h are shown the values of the membrane parameters deduced

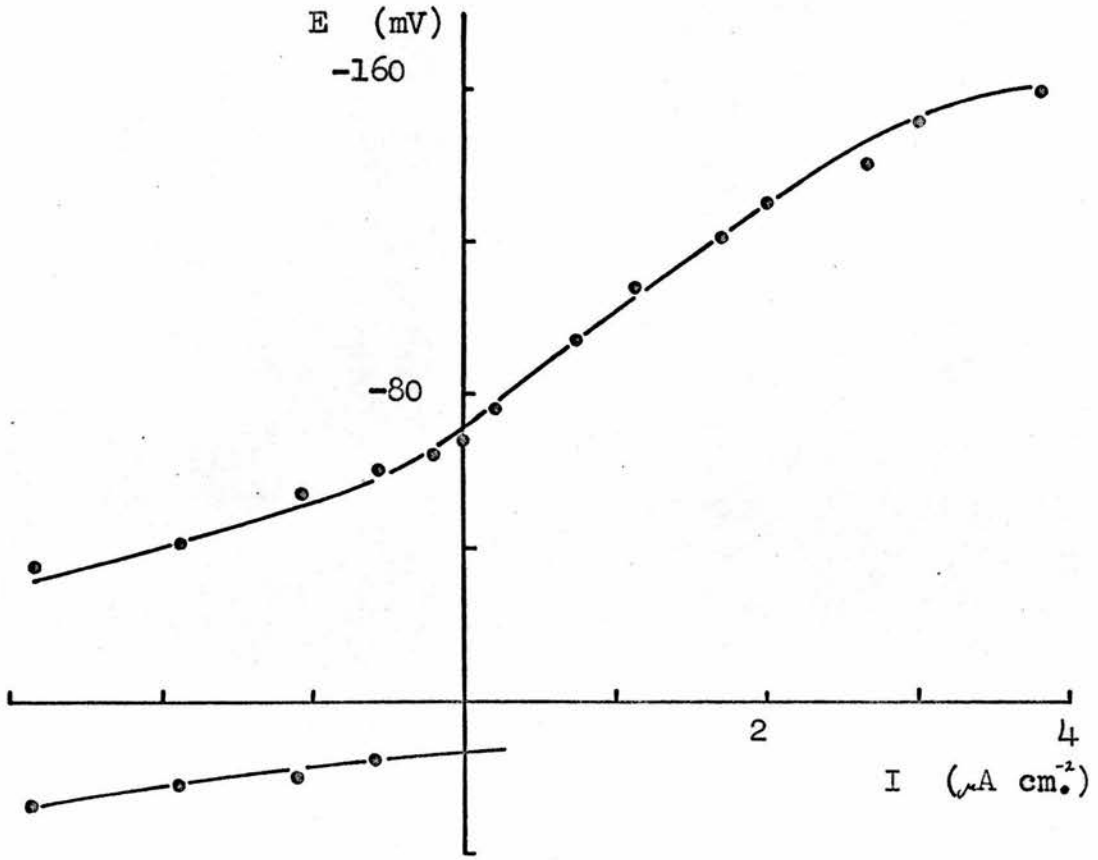


Fig. 3q Membrane characteristics with Ca-APW as external solution.

Table 3h. MEMBRANE PARAMETERS IN GAPW.

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm.)	R_e (KΩcm.)
1	-60	-15	15	10
2	-63	15	30	5·0
3	-60	23	6·7	10
4	-84	-37	30	6·6
5	-60	-20	27	12
6	-78	- 4	25	5·0
7	-70	10	33	6·7
8	-68	18	20	7·5
9	-76	-10	25	6·7
10	-64	- 1	12	2·5
11	-73	- 5	10	10
12	-74	24	10	2·0
13	-48	3	7	3·4
14	-65	16	11	4·1
15	-59	2	22	6·0
16	-83	0	25	3·2
17	-70	14	25	6·0
18	-75	6 5	22	2·2
19	-81	8	20	2·1
20	-6^0	27	15	8·3
Mean	- 69	4	22	5·9

Table 3i. MEMBRANE PARAMETERS IN HIGH Cl.

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm.)	R_e (KΩcm.)
1	-66	-13	16	10
2	-65	16	20	3·3
3	-64	6	40	2·2
4	-69	-43	20	13
5	-60	- 1	33	11
6	-83	-18	20	6·7
7	-50	18	20	6.1
8	-70	16	20	5·0
9	-75	- 4	36	10
10	-82	- 7	20	6·6
Mean	-68	- 3	28	7·4

Table 3j MEMBRANE PARAMETERS IN LOW Ca.

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm.)	R_e (KΩcm.)
1	- 90	-43	13	8·3
2	- 60	24	20	10
3	- 85	-29	26	8·0
4	- 80	-20	30	10
5	- 88	-17	40	6·7
6	- 100	-34	25	8.6
7	- 70	- 1	40	13
8	- 100	-14	26	8·0
9	- 97	-11	26	12
10	- 104	-36	35	12
Mean	- 87	-18	28	9·7

Table 3k. MEMBRANE PARAMETERS IN MEDIUM Na AND K.

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm².)	R_e (KΩcm².)
11	-80	19	14	2·2
12	-72	17	16	1·8
13	-44	17	10	4·0
14	-60	16	12	4·3
15	-64	10	22	5·4
16	-78	5	25	7·0
17	-58	16	17	7.1
18	-73	20	19	4.2
19	-92	-1	25	5·0
20	-74	28	11	3·0
Mean	-69	14	17	4·4

Table 31. MEMBRANE PARAMETERS IN HIGH K.

Exp.	E_m (mV)	E_e (mV)	R_m (K cm.)	R_e (K cm.)
11	-86	25	13	3·4
12	-59	53	9	2·0
13	-45	15	10	5·1
14	-65	14	18	6·2
15	-68	12	30	4·0
16	-94	4	20	3·0
17	-72	20	15	4·0
18	-66	36	18	3·5
19	-96	9	21	3·5
20	-78	44	16	3·2
Mean	-73	21	17	3·8

from the experiments in Ca APW on the two batches of the 10 cells.

High Cl and Low Ca as external solution.

It was found that neither of these solutions had any noticeable effect on the general shape of the characteristics obtained for the cell bathed in Ca APW. However, on average, when the cell was bathed in the Low Ca solution, E_m and E_e became more negative by 20 mV and 16 mV respectively. This is much the same effect as was observed in the voltage-clamp experiments. Typical current voltage characteristics are shown in Fig. 3r., and the membrane parameters obtained from experiments performed on 10 cells are shown in Tables 3i. and 3j.

Medium Na and K, and High K as external solution.

As in the voltage-clamp experiments it was difficult to distinguish between the characteristics for these solutions and for Ca APW (Fig. 3s.). The membrane parameters for experiments on 10 cells are shown in Tables 3k. and 3l.

Experiments at High Levels of Hyperpolarisation.

The relation between current and the potential of the membrane below the resting membrane potential is shown in Fig. 3t. It can be seen that the dynamic membrane resistance steadily decreases with decreasing membrane potential. This is in contrast to the curves obtained by Coster (1965); according to his results the dynamic membrane resistance is constant down to a potential level about 150 mV below the resting potential and at this level the dynamic membrane resistance drops abruptly to zero making the membrane potential independent of any further increase in membrane current. Removing Ca from the external solution had no effect on the shape of the current

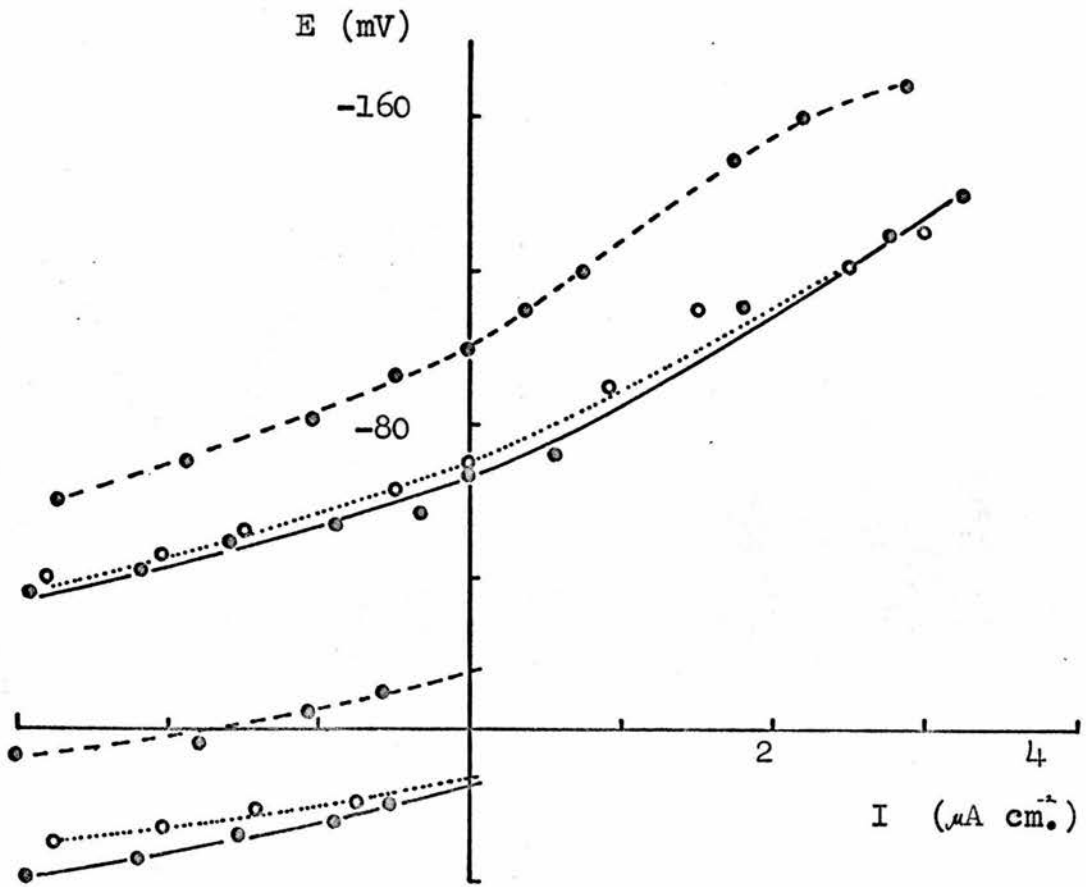
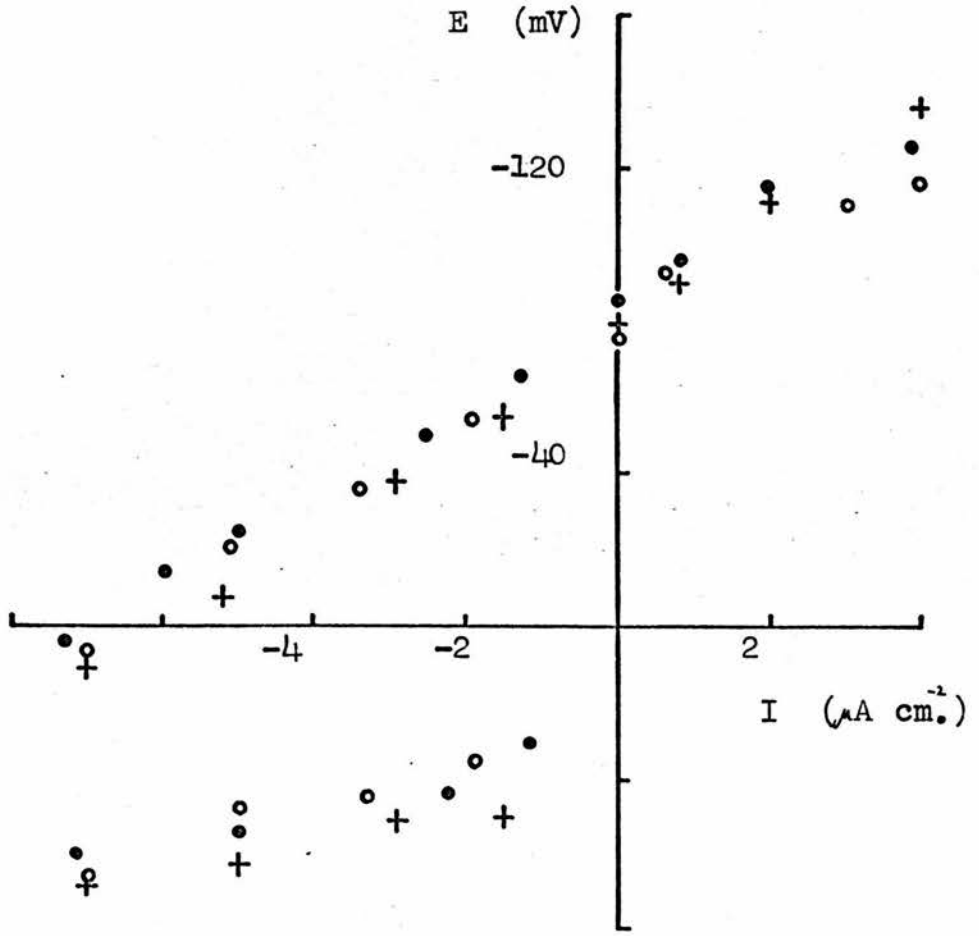


Fig. 3r Membrane characteristics in Ca-APW (full curve), in High Cl (dotted curve) and in Low Ca (dashed curve).

Fig. 3s The relation between membrane potential and membrane current in Ca-APW (closed circles), in Medium Na and K (open circles) and in High K (crosses).



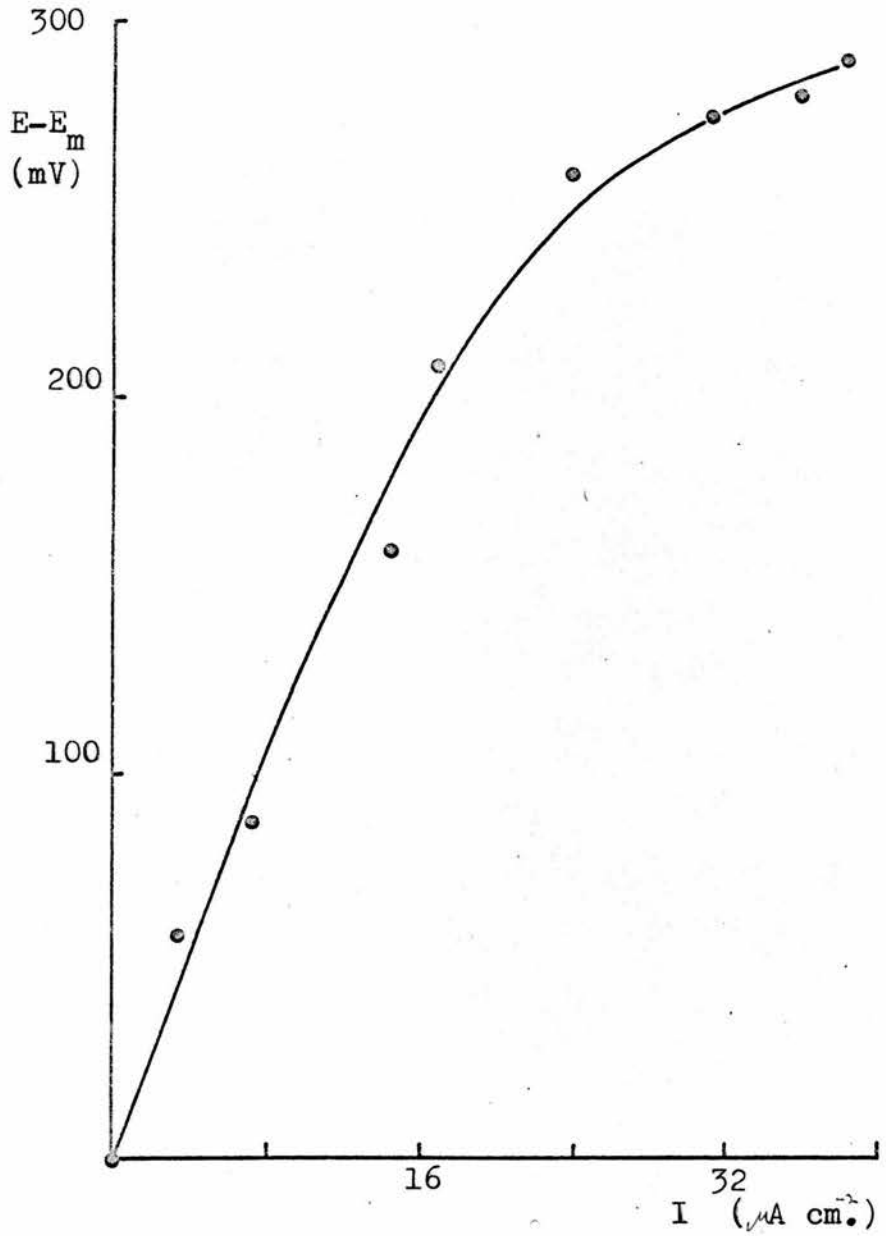


Fig. 3t The relation between membrane hyperpolarisation and membrane current.

voltage curve.

It must be mentioned that if these large currents were kept flowing through the cell for more than about 20 seconds, this normally had the effect of killing the cell. Further potential transients could last up to 10 seconds so that it was necessary to take some readings when the potential was not quite steady. These transients were always such that the initial membrane potential, following the injection of a square pulse of current, was always more negative than the final steady potential. However, with a given current flowing across the cell the membrane potential was usually read as soon as it became reasonably steady. The casualty rate in this experiment was about 50%.

3.34 Discussion

Resting and Excited States

The average value of the membrane resistance for 20 cells bathed in Ca APW was 20 KΩcm^{2}. and this is in fair agreement with the value obtained from the voltage-clamp experiments described in the previous section; the value of 5·9 KΩcm^{2}. for R_e compares well with 5·0 KΩcm^{2}. obtained from the voltage-clamp experiments. The shape of the current voltage curves is much the same except for the fact that it was not possible to obtain the complete curve for the membrane in the excited state by this method. This is the main drawback involved in studying the membrane by means of constant current.

The effect of Ca supports the observations and conclusions of the previous section; the cell wall is thus the main barrier to determining by a direct electrical method exactly which ions

carry the membrane currents in both the resting and excited states.

It has been mentioned that the process of excitation may usefully be regarded as the transition of the membrane from the resting state through a series of intermediate states to the excited state; and this is then followed by a further transition of the membrane from the excited state through another series of intermediate states to the resting state again. It is possible to construct the current voltage curves for each of these intermediate states using the current response of the membrane to square voltage pulses. However, when the membrane current is kept constant the potentials of the membrane in the first series of intermediate states are obscured by the potential transients due to the charging of the membrane capacitance; thus the characteristics of the intermediate states are more difficult to obtain by means of current-clamp experiments.

Three different types of transient potential were observed. The first type which was only sometimes observed will not be discussed, because, as already mentioned, it seems probable that this was caused by the injection of a hyperpolarising pulse during the refractory period. The other two transients, one at the beginning of a large hyperpolarising pulse and the other following the pulse, seem to be more fundamental. The first of these two transients will be called the 'anode-make' transient and the second the 'anode-break' transient; thus, the anode-break transient can lead to anode-break excitation, a phenomenon observed in nerve ~~but, as far as the present author~~ and (see Fundlay, 1959)

~~is aware, not previously~~ in Nitella. The explanation of anode-break excitation in nerve seems very reasonable, considering the amount of information available on the dependence of the Na and K conductances on the membrane potential. It seems clear that the explanation of this phenomenon in Nitella should be similar; however, such an explanation is difficult to give for Nitella, owing to the uncertainty regarding what happens to the membrane conductances during the action potential. Nevertheless, there seem to be two simple possibilities; either hyperpolarisation produces an increase in Cl conductance or a decrease in ~~either~~ the K ~~or Na~~ conductance. Thus when the current is switched off the membrane potential will be more positive than the resting potential; this potential then decays as the potential returns to the resting value, or if the anode-break transient is large enough, excitation will ensue. Assuming this explanation is correct, then it is clear that the increased Cl conductance or the decreased ~~Na or~~ K conductance must gradually return to the resting values when the current is switched off. Similarily, when the hyperpolarising current is switched on, either the Cl conductance will gradually increase ~~or Na~~ or K conductance will gradually decrease; thus there must be an initial potential transient whose peak potential is more negative than the steady potential, and this is exactly what is observed. Thus the phenomena of the anode-make transient, the anode-break transient and anode-break excitation are all explicable in the same manner as anode-break excitation in nerve. It remains to consider whether Cl or ~~Na or~~ K is responsible. It is not possible to clearly decide this without

performing flux experiments; in principle it should be possible to decide from the effect of Cl, ~~Na and~~ K on the current voltage characteristics if it were not for the fact, as has been pointed out already, that the cell wall would mask any such effect. No effect can be observed from the curves obtained either at constant current or at constant voltage. However, the fact that the membrane resistance decreases up to very high levels of hyperpolarisation, is evidence in support of the idea that the Cl conductance increases.

Since the anode-make and anode-break transients are clearly plasma membrane phenomena, it should be possible to learn something of the nature of the membrane from them. Since during these transients one (or more) of the membrane conductances is changing, the question as to how this can happen must be raised. There seems to be only two possibilities; either the ion mobilities or the ion concentrations are changing inside the membrane. It hardly seems possible that the ion mobilities could be changing, so that ion concentration changes are probably involved. The ion concentrations in the membrane are not known but during the anode-make transient the membrane current is around 2 μA cm^{-2}, which means that, at most, the ion concentration could be changing at a rate of 1·0 coul.sec^{-1}. which corresponds to 10 mM sec^{-1}. One would expect the ion concentrations in the membrane to be a lot less than 10 mM, for if the ion mobilities for the membrane were the same as for the external solution, then the ion concentrations in the membrane would be 10^{-10} mM. However, it is difficult to believe that only an extremely small fraction of the current is involved

in changing the membrane conductances, for it is probable that the transport numbers of Cl, K and Na are each greater than 0·01. Thus it must be concluded that the ion concentrations in the membrane are extremely high, comparable with the concentrations in the solutions, and that the ion mobilities are extremely low, probably a million times less than those of ions in water. Further evidence for this comes from the fact that the resistance of the membrane calculated from the membrane fluxes of K and Na is about 10 times larger than the measured value (Williams, Johnston and Dainty, 1964). This is explicable if the membrane is assumed to be composed of at least one layer of a-type and one layer of c-type exchange membrane. This is a clear example of a membrane whose internal ion concentrations, and hence conductances,can be changed by the passage of electric current. The flux of a particular ion through this membrane (when no current is flowing) will be controlled by that region of the membrane in which it is a minority carrier, but the total membrane resistance will depend on the concentration of majority carriers at all points in the membrane. Thus the ratio of the measured resistance to the resistance calculated from the ion flux measurements is a measure of the ratio of the concentration of majority carriers to minority carriers. But this ratio also determines the ratio of ions passing right through the membrane, when a current is passing, to the total number carried by the current. Thus one tenth of the current carries ions right through the membrane while nine tenths carries ions into or out of the membrane and thus is used to change the membrane ion concentrations. As has been

mentioned already 1·0 μA cm^{-2}. corresponds to 10 mM l^{-1}. sec^{-2}. Thus 10 mM liter^{-1} of an ion in the membrane can have a significant effect on the ion membrane conductance, so that the membrane ionic conductances must be even greater than this value. The idea that the membrane ionic concentrations are high derives support from considerations of the membrane structure. If the bimolecular lipid leaflet has a layer of protein on either side, it is reasonable that these protein layers could behave as ion exchange membranes and since the concentration of ions in the membrane must then be comparable with the concentration of protein molecules in the membrane, this would make the concentration about 1·0 M. Thus it can probably be concluded that the ion concentration in the membrane is of the order of 100 mM, which is surprisingly large. This agrees with the value required to predict the membrane 'punch-through' voltage, (Coster, 1965) from an equation relating membrane thickness, dielectric constant, ion concentrations and punch-through voltage.

Experiments at High Levels of Hyperpolarisation

The results of these experiments are not quite in agreement with those of Coster (1965); this may be due to the fact that Coster's method of performing the experiment was somewhat different. He plotted the current voltage curve by allowing the membrane current to increase linearly with time and recording the membrane potential on a moving chart. Coster gives no indication of the time taken to complete the scan of the curve; this is very important, because if the scan is completed within about half a minute, the recorded membrane potentials may only be transients. In fact Coster makes no mention of ever having

encountered any potential transients, nor does he make any mention of the time for which the membrane may be held at a high level of hyperpolarisation without damage to the cell. Currents of up to 50 μA cm.$^{-2}$ are passed across the membrane in this type of experiment and this is capable of heating the membrane, neglecting heat losses, at a rate of about 4·0 °C sec.$^{-1}$; this could damage the cell membrane in a short time and is possibly the reason why the present author found that the cells could be easily killed by these large currents. Another possible reason for the lack of complete agreement is that Coster's experiments were performed on another Nitella species.

Coster's model of the membrane is probably far too simple to explain the existence in the membrane of a stable resting and unstable excited state; nevertheless there may be some regions of the membrane which correspond to a sandwich of a-type and c-type material so that at appropriate potentials, punch-through could occur. It was concluded earlier that it was most likely that when the membrane was hyperpolarised the Cl conductance increased. This would also cause the dynamic membrane resistance to decrease with increasing hyperpolarising current, but probably not so abruptly as observed by Coster. It is possible that the increase in Cl conductance may be due to a c-a junction effect.

Conclusion

The experiments have shown that the current voltage curves obtained at constant current are in agreement with those obtained at constant voltage. The observed phenomena of the 'anode-make' transient, the 'anode-break' transient, anode-

break excitation and the gradual decrease in dynamic membrane resistance with increasing membrane hyperpolarisation are explicable if the membrane Cl conductance increases with increasing membrane hyperpolarisation. Cl conductance is most likely to depend on the membrane potential if the membrane resembles a multilayer structure of a-type and c-type material. The ion concentrations in the membrane are probably of the order of 100 mM.

3.4 MEMBRANE TRANSITIONS AT CONSTANT INWARD CURRENT.

3.41 Introduction

In the previous section membrane activity was studied at constant current, and it was pointed out that the main disadvantage of constant current experiments lay in the fact that membrane activity could only be studied at constant outward current. To be more precise, in order that the membrane make a transition to the excited state the stimulating current has to be outward; it thus follows that if the membrane current is to be held constant during the whole period of activity, then that current must be outward. However, since the membrane time constant during excitation is shorter than the time the membrane spends in the excited state it is clearly possible to inject a pulse of inward current at the peak of activity, thus hyperpolarising the membrane while it is in the excited state. If this current pulse has a long duration then the membrane will have to undergo a transition to the resting (hyperpolarised) state at constant current. In this way it is possible to observe the transition from the excited state to the resting state (but not from the resting state to the excited state) at constant inward current. These transitions are studied in this section.

Short pulses of hyperpolarising current have been injected into toad nodal membrane in the excited state by Tasaki (1956) and into squid giant axon membrane in the excited state by Tasaki and Hagiwara (1957) and for both cases it has been found that this has the effect of abolishing the remainder of activity and the refractory period. Attempts to find the same

phenomenon in Nitella were not successful.

3.42 Experimental Methods.

The electrode system and the potential recording and current recording circuit were the same as in the previous experiments. Current was injected through a high resistance (10 MΩ) but the polarity of the current could be switched from any desired value of inward current to any desired value of outward current (or the reverse) by means of a single switch and two potentiometers.

The operation of the system was as follows. With zero current flowing across the membrane, one potentiometer is arranged to supply enough current to stimulate the cell while the other is arranged to supply the constant hyperpolarising current; this hyperpolarising current is varied throughout the experiment. The stimulating current is then switched on and when the membrane has reached the peak of activity the inward current is switched on. The membrane current and voltage profiles were displayed on the oscilloscope and photographed. After about ten minutes this sequence of operations was repeated with a different hyperpolarising current.

The experiments were all performed with Ca-APW as the external solution.

3.43 Results

A typical series of potential responses to the injected current pulses is shown in Fig. 3u. The first set of traces shows the potential response to the (stimulating) pulse of outward current; this is merely the action potential profile with the ohmic potential drop due to the membrane current

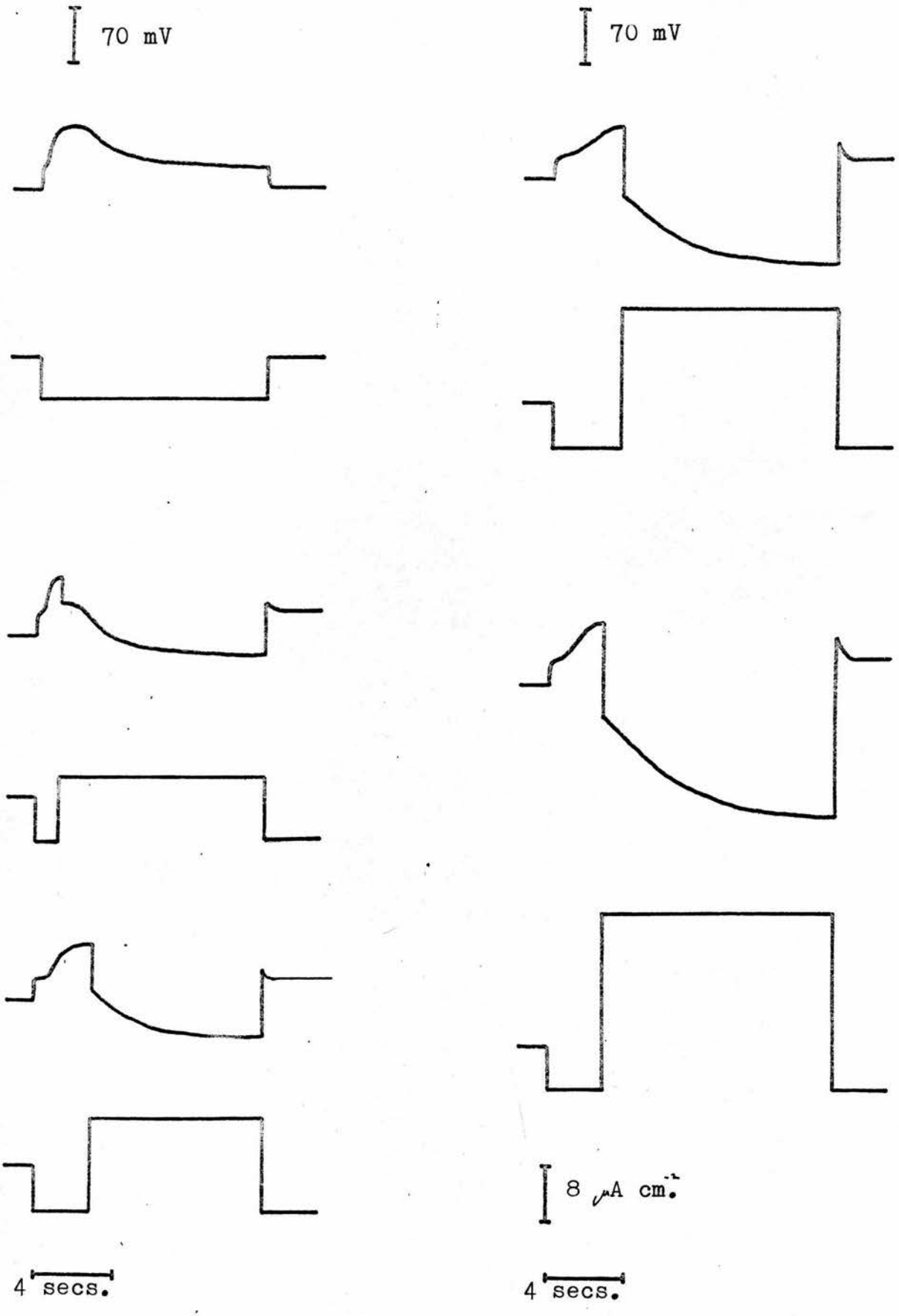


Fig. 3u Membrane transitions at constant current.

superimposed; this type of response was studied in the experiments described in the previous section. The next set of traces shows the potential response when a small inward current is superimposed upon the (stimulating) outward current at the peak of activity. The inward current abruptly drives the membrane potential of the excited membrane in the direction of the resting potential level; the membrane then remains in the excited state at this new potential level for about a tenth of a second and then makes a transition to the resting state. Since this transition is accomplished when the membrane current is inward, when the transition is completed the membrane will be hyperpolarised. When the inward current is switched off, the membrane is once more slightly depolarised, by the steady outward current. This outward current coupled with the act of switching off the inward current sometimes had the effect of producing another action potential.

The next three traces show the potential response for different and increasing values of the inward current, the outward current being the same in each case. With very large inward current the potential of the membrane in the excited state can be more negative than the resting potential, in other words more negative than the excitation threshold potential, and yet this in no way prevents the membrane making a gradual transition to the resting state. In nerve (Tasaki, 1956) the membrane activity is abruptly abolished if the membrane in the excited state is driven to a potential close to the threshold potential.

From these responses it is possible to construct the current

Table 3m MEMBRANE PARAMETERS IN APW

Exp.	E_m (mV)	E_e (mV)	R_m (KΩcm^2)	R_e (KΩcm^2)
1	-75	4	15	3·5
2	-64	8	13	6·3
3	-52	3	10	3·4
4	-66	8	6·0	4·5
5	-72	22	10	1·4
6	-73	38	18	6·4
7	-68	0	17	6·5
8	-63	58	12	2·4
9	-60	5	10	2·6
10	-66	- 1	11	3·4
11	-65	- 7	8 4	5·3
Mean	-66	13	12	4·2

voltage characteristics of the membrane. The variation of the peak of membrane activity with the inward membrane current gives the characteristic for the excited state. The variation of the steady membrane potential with inward current gives the characteristic for the resting state. As is to be expected these characteristics (Fig. 3v.) are similar to those obtained from the voltage-clamp experiments; in this particular example the values obtained for R_m and R_e were 15·0 and 3·5 KΩcm. The membrane parameters deduced from experiments on 12 cells are listed in Table 3m. However, the current voltage characteristic of the membrane obtained by the present method differs from that obtained from the voltage clamp experiments in one fundamental aspect. The excited state characteristic extends into the region where the excited state is more negative than the excitation threshold potential. According to the results of Kishimoto (1964) this cannot happen and in this region the membrane has a dynamic negative resistance.

It is possible using this method to construct all the current voltage characteristics of the membrane states which are intermediate between the resting and excited states; it is not possible to accomplish this with the voltage-clamp technique. This has not been done here; however, it can be very simply deduced from the potential profile of the membrane, as it makes a transition from the excited state to the resting state, that these intermediate state characteristics extend into the region where the potential is very much more negative than the resting potential.

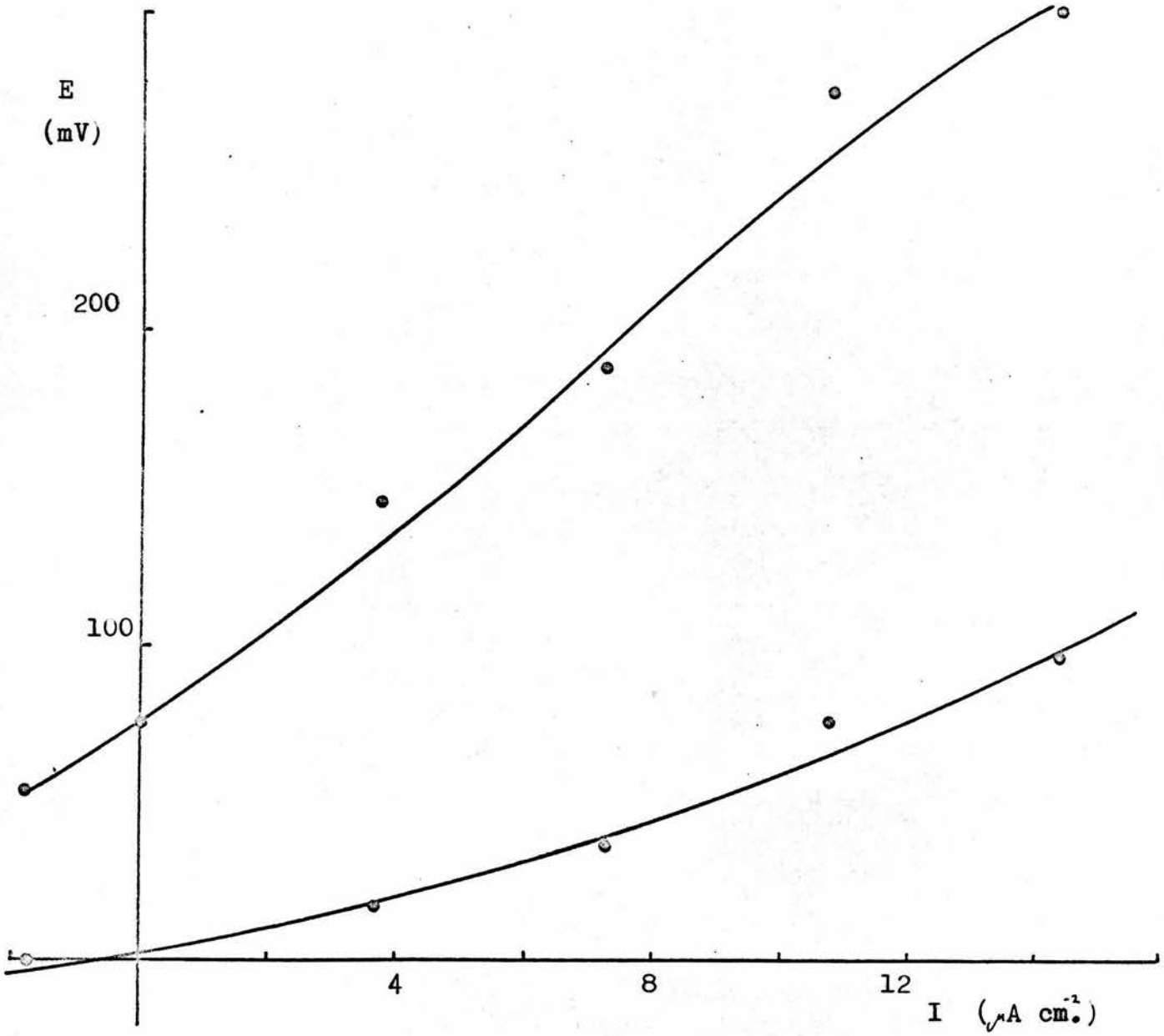


Fig. 3v Membrane characteristics with Ca-APW as external solution.

3.44 Discussion

The membrane parameters measured in the experiment agree fairly well with those obtained by the voltage-clamp and the current-clamp methods; the membrane resistances seem to be smaller than those encountered in the other experiments. There seems to be no simple explanation for this and it is probably due to the fact that the cells used had just been freshly collected.

The fact that the large inward currents used during these experiments did not abolish the membrane excitation shows that the membrane is different in this respect from toad nodal membrane and squid nerve, but although the present author did not observe abolition of the action potential in Nitella this does not mean that it does not occur. It is possible that with greater hyperpolarisation of the membrane in the excited state, the effect will be observed. Since Tasaki (1956) believes that the abolition of the action potential (and the ensuing refractory period) by a strong hyperpolarising pulse is evidence against some aspects of the sodium theory of nerve excitation, it cannot be adequately stressed that a definite answer to this question as to whether abolition of the action potential in Nitella is possible or not is of fundamental importance.

Further, the curve for the excited membrane passes through the region where the potential is more negative than the excitation threshold potential. This is not the case in nerve and Kishimoto has found that the membrane has a very low dynamic negative resistance in this region using the voltage-clamp method. Thus it appears that when this region of the

characteristic is studied at constant current the resulting curve is different from that obtained from a study at constant voltage; possibly the membrane is dependent on its past history. The cause of this is probably very fundamental, but since very little is known at present about the time course and potential dependence of the membrane Cl conductance, it is very difficult to explain the phenomenon.

Here the matter must rest for the time being; time has not permitted a more thorough investigation, but the present author is of the opinion that this type of experimental approach could be further exploited to advantage.

3.5 CONCLUSIONS

The experiments described in this chapter have all been concerned with the current voltage relations of the composite membrane consisting of the cell wall, the cytoplasmic membrane and the vacuolar membrane in series. It has been suggested that when the external ion concentrations are changed the effect on the resting potential is due to a potential difference across the cell wall. In contrast the transient and steady potential changes which occur when a current is passed across the composite membrane are very probably due to the cytoplasmic membrane. Throughout this chapter the action potential has also been assumed to be a cytoplasmic membrane phenomenon, and no evidence has been found which could invalidate this assumption.

The phenomenon of excitability has been regarded as a transition between the resting state of the (composite) membrane and an excited state, this transition then being followed by a further transition back to the resting state. The resting state membrane resistance and the excited state membrane resistance are on average 21 and 5·1 $K\Omega cm^2$ respectively; the membrane potentials in the resting and excited state have the same dependence on the ionic concentrations of the external solution (particularily those of Ca) and in Ca APW the values are on average -64 and +7 mV respectively.

The current voltage relations of the membrane for the two states have been obtained both at constant voltage and at constant current and the curves obtained by the two methods are in good agreement with one another. The potential profiles of

membrane transitions at constant current and the current profiles of membrane transitions at constant voltage have also been recorded. The phenomena of 'anode-make' and 'anode-break' transients and anode-break excitation have been discovered, and have been tentatively explained on the basis of the Cl hypothesis. Punch-through has not been observed but the dynamic membrane resistance does gradually decrease with increasing membrane hyperpolarisation; again, abolition of the action potential by means of large hyperpolarising pulses has not been observed but this is considered to be due to the pulses not being large enough. Finally, from a consideration of transient membrane phenomena, it has been estimated that the ion concentrations in the membrane are of the order of 100 mM l^{-1}.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. E. J. Williams for his helpful advice and constant encouragement during the course of this work. I would also like to thank Mr. J. Hogg for his stimulating discussions about the theory of transmission lines. Professor R. Brown must be thanked for placing the facilities of the Department of Botany at my disposal during the later part of the work.

I am indebted to Mr. J. Tait, formerly of the Biophysics Department Workshop and to the staff of the Workshop of the Department of Natural Philosophy for their invaluable technical assistance.

During the years 1964 - 1966 the author held a Northern Ireland Ministry of Education Research Studentship.

REFERENCES

Adelman, W.J., Jr., and Moore, J.W. (1961). Action of external divalent ion reduction on sodium movement in the squid giant axon. J. Gen. Physiol., 45, 93-103.

Baker, P.F., Hodgkin, A.L. and Shaw, T.I. (1962). The effects of changes in internal ionic concentrations on the electrical properties of perfused giant axons. J. Physiol., 164, 355-374.

Boyle, P.J. and Conway, E.J. (1941). Potassium accumulation in muscle and associated changes. J. Physiol., 100, 1-63.

Cole, K.S. (1949). Dynamic electrical characteristics of the squid axon membrane. Arch. Sci. Physiol., 3, 253-258.

Cole, K.S. (1965). Electrodiffusion Models for the Membrane of Squid Giant Axon. Physiol. Rev., 45, 340-379.

Cole, K.S. and Curtis, H.J. (1938). Electric impedance of Nitella during activity. J. Gen. Physiol.,22, 37-64.

Cole, K.S. and Curtis, H.J. (1939). Electric impedance of squid giant axon during activity. J. Gen. Physiol., 22, 649-670.

Coster, H.G.L. (1965). A quantitative analysis of the voltage-current relationships of fixed charge membranes and the associated property of 'punch-through'. Biophysic. J., 5, 669-686.

Curtis, H.J. and Cole, K.S. (1940). Membrane action potentials from the squid giant axon. J. Cell Compt. Physiol., 15, 147-157.

Dainty, J. (1962). Ion transport and electrical potentials in plant cells. Ann. Rev. Plant Physiol., 13, 379-402.

Dainty, J. and Hope, A.B. (1959). Ionic relations of cells of Chara australis. 1 Ion exchange in the cell wall. Austr. J. Biol. Sci., 12, 395-411.

Dainty, J., Hope, A.B. and Denby, C. (1960). Ionic relations of

cells of Chara australis. 11 The indiffusible anions of the cell wall. Austr. J. Biol. Sci., 13, 267-276.

Danielli, J.F. and Harvey, E.N. (1935). The tension at the surface of mackerel egg oil with remarks on the nature of the cell surface. J. cell compt. Physiol., 5, 483-494.

Davson, H. and Danielli, J.F. (1952). The Permeability of Natural Membranes. Cambridge. C.U.P. 2nd. Edn.

Diamond, J.M. and Solomon, A.K. (1959). Intracellular potassium compartments in Nitella axillaris. J. Gen. Physiol., 42, 1105-1121.

Dodge, F.A. and Frankenhaeuser, B. (1958). Membrane currents in isolated frog nerve fibre under voltage-clamp conditions. J. Physiol., 143, 76-90.

Engbaek, L. and Hoshiko, T. (1957). Electrical potential gradients through frog skin. Acta Physiol. Scand., 39, 348-355.

Findlay, G.P. (1959). Studies of action potentials in the vacuole and cytoplasm of Nitella. Aust. J. Biol. Sci., 12, 412-426.

Findlay, G.P. (1962). Calcium ions and the action potential in Nitella. Aust. J. Biol. Sci., 15, 69-82.

Findlay, G.P. (1964). Ionic relations of cells of Chara australis. V111 Membrane currents during a voltage-clamp. Aust. J. Biol. Sci., 17, 388-399.

Findlay, G.P. and Hope, A.B. (1964a). Ionic relations of the cells of Chara australis. V11 The separate electrical characteristics of the plasmalemma and tonoplast. Austr. J. Biol. Sci., 17, 62-67.

Findlay, G.P. and Hope, A.B. (1964b). Ionic relations of Chara australis. (IX Analysis of transient membrane currents ~~V11 The separate electrical characteristics of the plasmalemma and tonoplast~~) Austr. J. Biol. Sci., 17, ~~62-77~~ 400-411.

Finkelstein, A. (1964). Carrier model for active transport of ions across a mosaic membrane. Biophysic. J., 4, 421-440.

Finkelstein, A. and Mauro, A. (1963). Equivalent circuits as related to ionic systems. Biophysic. J., 3, 215-236.

Frankenhaeuser, B. and Hodgkin, A.L. (1957). The action of calcium on the electrical properties of squid axons. J. Physiol. 137, 218-244.

Friche, H. (1933). The electric impedance of suspensions of biological cells. Cold Spr. Harb. Symp. quant. Biol., 1, 1.

Gaffey, C.T. and Mullins, L.J. (1958). Ion fluxes during the action potential in Chara. J. Physiol., 144, 505-524.

Goldman, D.E. (1943). Potential, Impedance and Rectification in Membranes. J. Gen. Physiol., 27, 37-60.

Gorter, E. and Grendel, F. (1925). On bimolecular layers of lipoids on the chromocytes of the blood. J. exp. Med., 41, 439.

Graham, J. and Gerard, R.W. (1946). Membrane potentials and excitation of impaled single muscle fibres. J. cell compt. Physiol., 28, 99-117.

Grendel, F. (1929). Über die Lipoidschicht der Chromocyten beim Schaf. Biochem. Z., 214, 231-241.

Harris, E.J. (1953). The exchange of frog muscle potassium. J. Physiol., 120, 246-253.

Hermann, L. (1899). Zur Theorie der Erregungsleitung und der elektrischen Erregung. Arch. Ges. Physiol., 75, 574-590.

Hodgkin, A.L. (1939). The relation between conduction velocity and electrical resistance outside a nerve. J. Physiol., 94, 560-570.

Hodgkin, A.L. (1951). The ionic basis of electrical activity in nerve and muscle. Biol. Rev., 26, 339-409.

Hodgkin, A.L. (1957). Ionic movements in giant nerve fibres. Proc. roy. Soc. B, 148, 1-37.

Hodgkin, A.L. and Horowicz, P. (1960). The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibres. J. Physiol., 153, 370-385.

Hodgkin, A.L. and Huxley, A.F. (1952a). Currents carried by Sodium and Potassium Ions through the Membrane of the Giant Axon of Loligo. J. Physiol., 116, 449-472.

Hodgkin, A.L. and Huxley, A.F. (1952b). The Components of Membrane Conductance in the Giant Axon of Loligo. J. Physiol., 116, 473-496.

Hodgkin, A.L. and Huxley, A.F. (1952c). The Dual Effect of Membrane Potential on the Sodium Conductance of the Giant Axon of Loligo. J. Physiol., 116, 497-506.

Hodgkin, A.L. and Huxley, A.F. (1952d). A Quantitative Description of Membrane Current and its application to Conduction and Excitation in Nerve. J. Physiol., 117, 500-544.

Hodgkin, A.L., Huxley, A.F. and Katz, B. (1952). Measurement of current voltage relations in the membrane of the giant axon of Loligo. J. Physiol., 116, 424-448.

Hodgkin, A.L. and Katz, B. (1949). The Effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol., 108, 37-77.

Hodgkin, A.L. and Keynes, R.D. (1955). The potassium permeability of a giant nerve fibre. J. Physiol., 128, 61-88.

Hodgkin A.L. and Rushton, W.A.H. (1946). The electrical constants of a crustacean nerve fibre. Proc. Roy. Soc., B, 133, 444-479.

Hope, A.B. (1963). Ionic relations of cells of Chara australis. VI Fluxes of potassium. Austr. J. Biol. Sci., 16, 429-441.

Hope, A.B. (1965). Ionic relations of cells of Chara australis. X Effects of bicarbonate ions on electrical properties. Austr. J. Biol. Sci., 18, 789-801.

Hope, A.B. and Findlay, G.P. (1964). The action potential in Chara. Plant and Cell Physiol., 5, 337-379.

Hope, A.B. and Walker, N.A. (1960). Ionic relations of cells of Chara australis. 111 Vacuolar fluxes of sodium. Austr. J. Biol. Sci., 13, 277-291.

Hope, A.B. and Walker, N.A. (1961). Ionic relations of Chara australis. 1V Membrane potential differences and resistances. Austr. J. Biol. Sci., 14, 26-44.

Huang, C., Wheeldon, L. and Thompson, T.E. (1964). The properties of lipid bilayer membranes separating two aqueous phases: Formation of a membrane of simple composition. J. Mol. Biol., 8, 148-160.

Ives, D.J.G. and Janz, G.J. (1965). Reference Electrodes. Academic Press, 1965.

Julian, F.J., Moore, J.W. and Goldman, D.E. (1962). Current voltage relations in the lobster giant axon membrane under voltage-clamp conditions. J. Gen. Physiol. 45, 1217-1237.

Keynes, R.D. and Martins-Ferreira, H. (1953). Membrane potentials in the electroplates of the electric eel. J. Physiol., 119, 315-351.

Kishimoto, U. (1959). Electrical characteristics of Chara corallina. Ann. Rep. Scient. Works, Fac. Sci. Osaka Univ., 7, 115-146.

Kishimoto, U. (1964). Current voltage relations in Nitella. Jap. J. Physiol., 14, 515-527.

Koefoed-Johnsen, V. and Ussing, H.H. (1958). The nature of frog skin potential. Acta Physiol. Scand., 42, 298-308.

Levi, H. and Ussing, H.H. (1948). The exchange of sodium and chloride ions across the fibre membrane of the isolated frog sartorius. Acta Physiol. Scand., 16, 232-249.

Ling, G. and Gerard, R.W. (1949). The normal membrane potential of frog sartorious fibres. J. cell compt. Physiol., 34, 383-396.

MacRobbie, E.A.C. (1962). Ionic relations of Nitella translucens. J. Gen. Physiol., 45, 861-873.

MacRobbie, E.A.C. and Dainty, J. (1958). Ion transport in Nitellopsis obtusa. J. Gen. Physiol., 42, 335-353.

Mauro, A. (1962). Space charge regions in fixed charge membranes and the associated property of capacitance. Biophysic. J., 2, 179.

Mullins, L.J. (1962). Effect of chloride ions during the action potential of Nitella. Nature, 196, 986-987.

Nernst, W. (1888). Zur Kinetic der in Lösung befindlichen Körper: Theorie der Diffusion. Z. Physik. Chem., 2, 613-627.

Oda, K. (1956). Resting and action potentials in Chara braunii. Sci. Rep. Tohoku Univ., 22, 167-174.

Oda, K. (1961). The nature of the membrane potential in Chara braunii. Sci. Rep. Tohoku Univ., 27, 159-168.

Ooyama, H. and Wright, E.B. (1961). Anode-break excitation on single Ranvier node of frog nerve. Amer. J. Physiol., 200, 209-218.

Osterhout, W.J.V. and Harris, E.S. (1928). Positive and negative currents of injury in relation to protoplasmic structure. J. Gen. Physiol., 11, 673-700.

Osterhout, W.J.V. and Hill, S.E. (1930a). Negative variations

in Nitella produced by KCl and chloroform. J. Gen. Physiol., 13, 459-467.

Osterhout, W.J.V. and Hill, S.E. (1930b). Salt bridges and negative variations. J. Gen. Physiol., 13, 547-552.

Osterhout, W.J.V. (1934). Nature of the action current in Nitella. 1 General considerations. J. Gen. Physiol., 18, 215-227.

Phillips, G.A. (1962). Status of the ion-exchange membrane fuel-cell. Electrical Engineering, 81, 194-201.

Plank, M. (1890). Über die Potential differenz zwischen zwei verdünnten Lösungen binärer Electrolyte. Ann. Physik. Chem., 40, 561-576.

Robertson, J.D. (1957). New observations on the ultrastructure of the membranes of frog peripheral nerve fibres. J. biophys. biochem. Cytol., 3, 1043-1048.

Scheer, B.T. and Mumbach, M.W. (1960). The locus of electromotive force in frog skin. J. cell compt. Physiol., 55, 259-266.

Spanswick, R.M. (1964). Ion uptake and electrical potentials in plants. Thesis, Univ. Edinburgh.

Spanswick, R.M. and Williams, E.J. (1964). Electric Potentials and Na, K and Cl concentrations in the vacuole and cytoplasm of Nitella translucens. J. exp. Bot., 15, 193-200.

Spanswick, R.M. and Williams, E.J. (1965). Ca fluxes and membrane potentials in Nitella translucens. J. exp. Bot., 16, 463-473.

Spanswick, R.M., Stolarek, J. and Williams, E.J. (in press). J. exp. Bot.

Tasaki, I. (1939). The electro-saltatory transmission of the

nerve impulse. Amer. J. Physiol. 127, 211-227.

Tasaki, I. (1956). Initiation and abolition of the action potential of a single node of Ranvier, J. Gen. Physiol., 39, 377-395.

Tasaki, I. and Hagiwara, S. (1957). Demonstration of two stable potential states in the squid giant axon under tetraethylammonium chloride. J. Gen. Physiol., 40, 859-885.

Taylor, G.W. (1942). The correlation between sheath birefringence and conduction velocity with special reference to cat nerve fibres. J. cell compt. Physiol., 20, 359-372.

Teorell, T. (1953). Transport processes and electrical phenomena in ionic membranes. In Progress in Biophysics, 3, ed. Butler, J.A.V. and Randall, J.T. London: Pergammon Press.

Teorell, T. (1949). Membrane electrophoresis in relation to bioelectrical polarisation effects. Arch. Sci. Physiol., 3, 203-219.

Umrath, K. (1930). Untersuchungen über Plasma und Plasmaströmung an Characeen. lV Potentialmessungen an Nitella mucronata mit besonderer Berücksichtigung der Erregungserscheinungen. Protoplasma, 9, 576-597.

Umrath, K. (1932a). Die Bildung von Plasmalemma (Plasmahaut) bei Nitella mucronata. Protoplasma, 16, 173-188.

Umrath, K. (1932b). Der Erregungsvorgang bei Nitella mucronata. Protoplasma, 18, 258-300.

Umrath, K. (1934). Der Einfluss der Temperatur auf das elektrische potential, den Aktionsström und die Protoplasmaströmung bei Nitella mucronata. Protoplasma, 21, 329-334.

Ussing, H.H. (1949). The distinction by means of tracers between

active transport and diffusion. Acta Physiol. Scand., 19, 43-56.

Vaidhyanathan, V.S. (1965). Statistical mechanical theory of electrolyte diffusion inside a charged membrane. J. Theoretical Biol., 8, 478-488.

Walker, N.A. (1955). Microelectrode experiments on Nitella. Austr. J. Biol. Sci., 8, 476-489.

Walker, N.A. (1957). Ion permeability of the plasmalemma of the plant cell. Nature, 180, 95.

Walker, N.A. (1960). The electric resistance of the cell membranes in a Chara and Nitella species. Austr. J. Biol. Sci., 13, 468-478.

Weidmann, S. (1951). Effect of current flow on the membrane potential of cardiac muscle. J. Physiol., 115, 227-236.

Williams, E.J., Johnston, R.J. and Dainty, J. (1964). The electrical resistance and capacitance of the membranes of Nitella translucens. J. exp. Bot., 15, 1-14.